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Wild Species-Hybrids in the Phanerogams. II
H. H. ALLAN 77

Cytology of Bacteria. II ..... GEORGES KNAYSI 106

FUTURE CONTENTS
ON INSIDE BACK COVER

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THE BOTANICAL REVIEW

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WILD SPECIES–HYBRIDS IN THE PHANEROGAMS. II[1]

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INTRODUCTION

When the previous article was published in 1937 it was necessary to argue that wild species-hybrids provide important material for taxonomic, genetic and evolutionary studies. The decade just past has shown a growing recognition of this, and important papers have appeared showing the value of combining studies of wild hybrids with work in the experiment garden. The upset of the war period has made it impossible, especially to one in an isolated country, to follow all the work in this field, and valuable contributions may have missed notice in this supplement. It is hoped, nevertheless, that the review will serve a useful purpose. The different fields of work have so interacted upon one another that it is no longer possible or desirable to confine discussion merely to field observations. The synopsis serves to show that there has been definite progress towards that desired rapprochment between workers in diverse fields.

FIELD OBSERVATIONS

These serve to provide indications of suitable subjects for more intensive studies. While no attempt is made here fully to cover the mass of observations the world over, it is hoped that a sufficiently representative selection has been given.

Field studies were published at various times in the decade on the following genera:

Agropyron × *Elymus* × *Hordeum* (196)
Agropyron × *Elymus* × *Sitanion* (195)
Agrostis (161), accepting the natural occurrence of *A. stolonifera* × *Polypogon monspeliensis.*
Alnus (56)
Ambrosia (172)
Anagallis (136, 154)
Aster (177)
Balsamorhiza (157)
Bromus (125), confirmed experimentally.
Carex (175)

[1] Supplement to article in The Botanical Review 3: 593–615. 1937.

Carmichaelia (179), no evidence of hybridism.
Ceanothus (141), degree of fertility of hybrids accepted as an index of relationship between the parental species.
Celmisia (138); (180), doubtful hybrid, apparently sterile, with *Olearia*.
Centaurea (163)
Centaurium (91)
Cephalanthera (160)
Chaenactis (199)
Coriaria (156), forming a complete linkage between the seven species recognized.
Cornus (26, 164, 165), yielding a diversity of fertile offspring.
Dactylorchis (197)
Danthonia (213)
Elymus × *Agropyron* × *Sitanion* (195)
Elymus × *Agropyron* × *Hordeum* (196)
Epilobium (198)
Epimedium (184), crossing with *Vancouveria* takes place in gardens but doubtfully in the wild.
Eucalyptus (28, 140)
Haastia (139)
Hevea (64)
Hordeum × *Agropyron* × *Elymus* (196)
Leucogenes (2), sterile hybrids with *Raoulia*.
Malus (142)
Mentha (119), regarding the Swedish forms of *M. gentilis* as "ein Schwarm von Abkömmlingen aus der Kreuzung *M. arvensis* × *spicata*".
Olearia (180), doubtful hybrid, apparently sterile, with *Celmisia*.
Oryzopsis × *Stipa* (120)
Oxalis (174), after prolonged search found no satisfactory evidence of natural hybridism in South African species, though opportunity by proximity is not infrequent.
Parthenium (169), 162 hybrids from immediate crosses in nature were identified from a greenhouse population of some 205,000 plants grown from seeds collected in the wild.
Pedicularis (171)
Poa (124)
Populus (172); (123), discusses controlled F_2 progeny derived from F_1 individuals arising from a natural cross between *Populus alba* and *P. grandidentata*.
Potamogeton (202)
Prunus (176), between an indigenous and a naturalized species.
Quercus (134)
Raoulia (2), sterile hybrids with *Leucogenes*.
Salix (71, 78)
Sitanion × *Agropyron* × *Elymus* (195)
Solidago (77, 95)
Sonchus (24)
Stipa (135) × *Oryzopsis* (120)
Vancouveria (184), crossing with *Epimedium* takes place in gardens but doubtfully in the wild.
Verbena (143)
Viola (198)

RELATION TO TAXONOMY

Whether the taxonomist has any concern with hybridization, either in the wild or in the experiment garden, has been debated. Bremekamp (26) argues that the taxonomist "is not interested in the origin, but in the characters of his plants". Allan (4) affirms that the study of wild hybrids is an essential aspect of taxonomic work and that purely observational work can yield information on material likely to be useful in more detailed and experimental studies. The widespread nature of wild hybridism in the flora of New Zealand, and the occurrence of multiform hybrid swarms is illustrated. Bremekamp (27) reiterates that for purposes of classification it is not the origin that counts but the character complex. Uittien (204) considers that for taxonomy hybrids do not exist, since taxonomy

and its nomenclature are based on morphology, not on parentage. Fosberg (82, 83) vigorously combats the views of Bremekamp and Uittien. He maintains that genetics is one of the fundamental bases of the new experimental taxonomy, with systematic hybridization as its most powerful tool. He considers that one who "refuses to indicate hybridism is either over-cautious, or has never worked on a group of plants which produces hybrid swarms". Gilmour and Turrill (92) insist that the taxonomist must as far as possible consider all the attributes of living things, of which hybridism is one. There should be a proper taxonomic treatment of hybrids. Murbeck (146) agrees that hybridization must be considered in the delimitation of species, and gives instances in *Arctium, Celsia, Potentilla, Verbascum* and *Viola.* Palmer (159) says that "the plea that species should be defined on the basis of genetic relationship is commendable. That is what taxonomy attempts to do".

Arwidsson (16) lists for the region studied hybrids in *Betula, Cerastium, Draba, Juncus, Ranunculus, Rubus* and *Salix.* He also admits seven hybrids in *Carex* (40 species present) but points out that many supposed *Carex* hybrids are abnormal forms attacked by *Cintractia.* Camp (31–34) discusses hybrids in *Gaultheria* and *Pernettya* (and between the two genera), hybrid swarms in *Befaria,* and the nomenclatorial problems involved. "Such so-called 'species' are met with all too frequently in the Ericales in such genera as *Befaria, Rhododendron, Gaultheria, Pernettya,* and *Vaccinium*". In *Crataegus* he advises caution until the genetic structure is better known. In a useful discussion of "The herbarium in modern systematics" he remarks, "The day of the taxonomist who putters alone in his herbarium with an other-worldly stare is done. He must shed his robe of academic classicism and seclusion, brush off the accumulated dust of the centuries, and come face to face with the dynamics of living populations".

Chaytor (37) lists 23 hybrid forms in *Lavandula* and remarks, "that morphologically different plants may have had the same species as their immediate or original parents is to be explained by the intra-specific variation of several of the more widespread species and by the probable occurrence of segregation and back-crossing". Cugnac (57–59) continued his studies on *Bromus,* reproducing a form that "has been extinct for about 50 years". Fassett (68), in populations of *Linaria,* found internal barriers to hybridization and

recognized only one doubtful hybrid. Fassett (71–75) also studied in some detail three species of *Juniperus,* including hybrid swarms. The examination of large collections of material showed that "Colonies in the east are pure *J. virginiana;* in Kansas occasional plants show a slight tendency toward *J. scopulorum;* in Nebraska this tendency is stronger; in the Bad Lands of South Dakota colonies show a completely scrambled mixture of characters of the two, then grade off to pure *J. scopulorum* in the west". The resultant complex is ascribed to recent and more ancient meetings. He recognizes two varieties of *J. virginiana* and two of *J. scopulorum,* but one in each species is perhaps a hybrid with *J. horizontalis.*

Griesinger and Klinkowski (106) record four spontaneous interspecific hybrids in *Ornithopus,* linking four of the five recognised species. Failure to produce hybrids experimentally is attributed to faulty technique. Howard and Manton (114) show that the wild triploid *Nasturtium officinale* is a hybrid between the wild diploid and tetraploid. The name *N. officinale* is restricted to the diploid, and a new name *N. uniseriatum* is given to the wild allotetraploid. Johnson (120) found that the type specimens of *Oryzopsis bloomeri* and of *O. caduca* show that these "species" are hybrids between *O. hymenoides* and *Stipa occidentalis* and *S. viridula,* respectively. Kramer (129), studying natural populations of guayule and mariola, found evidence of interspecific hybridization. Lewis and Epling (133) give field and experimental evidence that *Delphinium gypsophilum,* a constant diploid species, arose from the crossing of *D. recurvatum* and *D. hesperium.* Nannfeldt (147, 151) discusses a sterile hybrid between *Poa supina* and *P. annua* and its distribution.

Nygren (155) synthesized *Calamagrostis purpurea* both by colchicine treatment of *C. canescens* and by crossing *C. canescens* with *C. epigeios.* Apomixis is attributed to the combination of complementary genes in the hybrid zygote. Palmer (159) considers that hybrids undoubtedly occur in *Crataegus,* all species probably being potentially inter-fertile, but hybridization is greatly restricted by certain physiological and physical factors. Polunin (162) adopts a cautious attitude as regards supposed *Carex* hybrids, and a cautious attitude in general. Rollins (170) found that crosses linking five species of *Parthenium* were easily obtained. Stebbins

(190) gives evidence that *Bromus arizonicus* is an allopolyploid derived from the crossing of *B. henkeanus* and *B. trinii.* Stebbins and his co-workers (195, 196) describe artificial and natural hybrids in the Hordeae, the experimental supporting the field evidence. Intergeneric hybrids involving the linking of *Agropyron, Elymus, Hordeum,* and *Sitanion* are described. Nomenclatorial problems are discussed, and it is considered that the results indicate the need for a drastic revision of the taxonomy of the tribe.

Frandsen (86), crossing *Brassica campestris* and *B. nigra,* obtained amphidiploids. The data obtained supported the view that *B. juncea* may be an amphidiploid hybrid of natural origin. Stockwell and Richter (200) discuss the status of *Pinus attenuata* and *P. radiata* in view of the occurrence of fertile F_1 and F_2 hybrids. Camp (35) states that "no effective sterility barriers exist between the populations of *Cinchona* in the Andes of Ecuador"; where they now chance to meet, complete series of intergrades usually occur. Some have given rise to extensive secondary independently reproducing groups. There is an intergrading of both morphological and biochemical characteristics. Cleland (49) points out the difficulties that face the taxonomist dealing with *Oenothera,* and gives analyses of several "races" from the cytogenetic point of view. He considers that there is strong suggestion "that hybridization has played an important rôle in the production of the various races". Through natural crossing, diverse complexes have been brought together. Many of these hybrids have bred true, owing to the lethals present, and have become established as the true breeding races or species. We are confronted, therefore, with the possibility that a large percentage of the *Onagra* population is actually of hybrid origin.

Clausen and his co-workers (39–48) have made valuable contributions to "experimental taxonomy". Discussing the various degrees of incompatibility found in different species crosses, they conclude that a large percentage of the hybrids produced will be wiped out under natural conditions and that the few vigorous offspring left will largely drop back into one of the two original species. They agree that introgression may occur and that new amphidiploid species may arise. An amphidiploid origin through hybridization is postulated for a species of *Madia,* but amphidiploidy is considered to be a rare evolutionary path in the Madiinae. *Layia hieracioides*

was experimentally proved to be an amphidiploid. They recognize that "as modern genetics developed in this century, it was rediscovered that hybridity is not a rare phenomenon and that many hybrids between species are partially fertile". Systematic hybridization is accepted as a "powerful analytical tool", while the use of the terms "ecotype", "ecospecies" and "coenospecies" is deprecated where experimental evidence is lacking. A vigorous but completely sterile *Madia* × *Layia* hybrid occurred in a culture from seed collected from a wild population. Two amphidiploids, one of *Madia,* one of *Layia,* occurred spontaneously in the experiment garden. Four species of *Potentilla* appeared to be linked by natural hybrids. The thesis: "taxonomic relationship between forms is roughly proportional to their ability to exchange genes either directly or indirectly through an intermediary", is illustrated by examples from *Artemisia, Pentstemon* and *Zauschneria.* Two natural hybrids in a *Layia* population "duplicated in appearance and behaviour the much larger population previously produced in the experiment garden". Crosses within a group of 14 species of *Layia* indicated a mosaic relationship as to "the relative ease or difficulty with which genes can be transmitted back and forth from one species to another". The data are assembled in an excellent diagram (45). Three amphiploids in the Madiinae, arising from sterile F_1 hybrids, without chemical or physical treatment, were thoroughly analysed and indicate what can happen in the wild. The initiation of amphiploidy within a mature comparium tends to regenerate it. The conclusion is reached that "Evolution may be said to be reticulate in pattern from the level of the ecotype to that of the comparium, but beyond that level it becomes exclusively forked in type". *Aquilegia* was found to combine extreme morphological and ecological variability with a high degree of crossability. In general, "Ecologically and taxonomically the amphiploid reacts as an interspecific F_1 hybrid that has become constant", and "successful amphiploids arise from successful interspecific hybrids . . . if the amphiploid is to remain successful during succeeding generations, the original balance must remain unchanged". It is considered that successful amphiploids will arise only from hybrids between species "so distantly related that their chromosomes cannot pair with each other or only rarely do so".

Gregor (104, 105), as a result of hybridization experiments, con-

cludes "that, when given an opportunity to hybridize, the North American—North European—diploid Alpine population [comprising five recognised species of *Plantago*] constitutes a single intrafertile group, although in such a widespread population the potential gene exchange is not realized in nature owing to the spatial isolation of its parts". Taxonomic difficulties, arising as a result of gene exchange, lead Gregor to advocate that the experimental taxonomist should use a terminology based on the ecotype concept and not on the "present system of classification". He points out the rather frequent "misuse of the ecotypic terminology, particularly in botanical literature", and emphasizes the necessity that "The extensive, and in many respects promiscuous, collecting of museum material must perforce largely be replaced by an intensive, orderly and planned assembling of representative samples from the all-important breeding communities".

Experimental taxonomy, as it becomes developed and its principles clarified, seems destined to play an important part in that synthesis of results from varied lines of botanical research that to many botanists, and biologists in general, seems so necessary for real advance.

THE GENUS RUBUS

The genus *Rubus,* as treated by different workers, well illustrates the divergence of views on taxonomic principles and practice that still exists when genera of large size are being studied. Rydberg (173) recognised 112 species in the North American flora, 69 of which are not recorded as producing hybrids. For the remaining 43 species 90 hybrid groups are admitted (including three garden hybrids). Under this treatment we find a large comparium involving 28 species, in which *R. argutus* (12 crosses), *R. baileyanus* (10 crosses), *R. canadensis* (15 crosses), *R. nigrobaccus* (14 crosses), *R. procumbens* (12 crosses), play a large part in the linkage. Also the Arctici and Saxatiles are linked through *R. pubescens.* Rydberg by no means regarded all these hybrids as definitely established.

Bailey (22) gives a detailed discussion of the genus in North America. He recognises 390 species and a number of varieties, claiming that he has taken a broad view and has not split the species finely. Based on over 50 years of intensive study in the field, and a great mass of herbarium material, the monograph deserves close

study. Especially useful are the notes on field work and methods of collecting and description. He admits no hybrids and in his detailed treatment of the species pays but slight attention to those holding other views. He remarks, "It is not to be understood that I am opposed to recognition of hybrids in *Rubus*. My objection is to the unscientific assumption that if a specimen, be it ever so poor, cannot be referred to any species at that moment recognized it must perforce be a hybrid. One may find in the field what are apparently wild hybrids, but they appear to be no more common or any more puzzling than in any other large genus". He rightly says, "Hybridity is not an assumption, but a subject capable of investigation". Of Rydberg's treatment he says, "It carried the hybridity assumption to its highest point in this country". Bailey considers that the conclusions of Rydberg (he is surely incorrect in stating that Rydberg admitted 153 hybrids in 36 species of *Eubatus*) and Brainerd (not seen by me) "disclose the prevailing incompetence in *Rubus* and remove the native blackberries from taxonomic study. Other genera than *Rubus* begin to show the break-away from former assignments of miscellaneous hybridity". "First", he says, "we must learn the species, that we may have starting-points; then we may speculate on hybridity and other questions". But surely, in such a group, the problem of what are the species and what are hybrids must be studied *pari passu*, and present dicta on species need investigation as much as dicta on hybrids. Not speculation but experimental study will yield results of value.

Crane and Darlington (55) had concluded that "The possibilities of crossing among species of *Rubus* are evidently determined to a great extent by the purely numerical relations of their chromosome complements. Hybrids are known to occur between the most distant species, yet crossing is often difficult between those having the greatest affinity". Hegi (112) said, "Die Gattung *Rubus* ist mit mindestens 300, bei engerer Fassung des Artbegriffes mit über 3000 Arten fast über die ganze Erde verbreitet. So schwer ihre Sektionen und Arten zu gliedern sind, so gut ist sie selbst abgegrenzt". He admits 19 major species within the area covered by his "Flora", but most of these are collective species and are split up into smaller species, with a total of 87. Hybridism is accepted as prevalent, but space was not available for any full treatment. It is suggested that a number of species are of hybridogenous origin.

Gustaffson (107–109) accepts the occurrence of hybrids in the Scandinavian *Rubi*. For most of the Eubati "there is a great variation due to hybridization", so that in the different groups there is a large number of transitional forms, while a great many localised and endemic microspecies have arisen. Even several "good" species are heterozygous to a "surprisingly great extent". Crosses between them or even segregation products may therefore give origin to "floras" of blackberries so rich in biotypes that each limited area contains its own endemism. Allopolyploidy is declared to be common, and the occurrence of apomicts has been substantiated. We now "know the principle outlines of the European blackberry flora, its systematics and geography". Lidforss, states Gustaffson, had proved that the Corylifolii are primary and secondary cross products of the Moriferi (Eubati) veri and the tetraploid *R. caesius*. "The astounding confusion of local and widely distributed types is simply due to the occurrence of pseudogamy, *i.e.*, parthenogenetic embryo formation accompanied by sexual endosperm development, maintaining in nature heterozygous segregates and hybrid derivatives". Further, "The apomicts in *Rubus* have not lost their capacity of sexual seed production but take part in a prosperous equilibrum system of apomixis and sexuality".

Judging from published descriptions and from the apparent difficulty in ascribing "varieties" to a particular species, the Asiatic *Rubi* would repay the like intensive and experimental study. In the New Zealand flora five species of *Rubus* are recognized, some with heteroblastic development, and all subject to modification by habitat conditions. Wild hybrids are very rare, and apparently sterile, though capable of vigorous vegetative growth. An artificial hybrid has occasionally produced flowers, but these show various abnormalities, and so far no viable seed has been produced.

NOMENCLATORIAL QUESTIONS

These have received some attention during the past decade, without any general agreement being reached. That the treatment in the International Rules is in need of modification is evident from the discussions aroused. A vigorous controversy was entered upon between Miss M. L. Green (99, 100) and Furtado (88, 89), arising from the problem of the correct name of the yellow hybrid aster. The two authors came to quite divergent views on the

meaning and application of the Rules. Furtado comments that "there is no reason why systematic names of any sort of hybrids should receive a different treatment from the systematic names of non-hybrid taxonomic groups of corresponding rank", and suggests that "it should be recommended in the Rules not to give a systematic name to any hybrid, unless there are reasonable grounds for believing the hybrid to be stable or homozygous". Green considers "that there is every reason for according [the technical names of hybrids] different treatment". She points out the possibility of "confusion of thought between a genus and a generic name". Furtado makes a lengthy rejoinder and concludes that "the procedure adopted by Miss Green cannot be defended from the Rules of Botanical Nomenclature", and that if Miss Green's procedure "is the ideal one for naming hybrids it should be considered by the next Botanical Congress". Later Furtado agrees that "The Rules seem to be rather ambiguous on the status of the names of hybrids, and current practice is too contradictory to be of any use in expounding the correct application of the Rules". He gives several examples.

Allan (3) points out certain difficulties caused by the phenomenon of hybrid swarms. Numerous hybrid forms in the past have received specific names. Often what has been named represents only a small portion of a hybrid swarm. To extend the use of such names to cover all forms leads to confusion. "At present it is wisest to remain content with a formular treatment, and to treat the authority to provide names 'whenever it seems necessary or usual' as a privilege to be exercised with the utmost restraint". Sprague (183) considers that Allan has not distinguished between a group and its name. He suggests that names that are actually telescoped formulae should be restricted to hybrids whose origin has been proved experimentally.

Cain (29) points out that names of hybrids bestowed according to the International Code may be quite ambiguous in their application. He illustrates by reference to Oliver's method of naming hybrids in *Dracophyllum, e.g.,* ×*D. erectum* (*D. prostratum* ×*rosmarinifolium*). "The geneticist, ecologist, or geographer wonders what kinds of hybrids such names represent . . . one wonders how a taxonomist can draw up a description to cover a natural hybrid swarm and, if his description is based on one of a few hybrid

forms, whether he realizes that he has entered on an almost endless descriptive task".

Uittien (204) considers that the words "hybrids" and "half-breeds" should be suppressed from the Rules. For the taxonomist, he considers, hybrids do not exist. But hybrids exist in nature, and it is difficult to see how the taxonomist can avoid considering them, unless, indeed, he does as Uittien suggests and relies purely on his eyes. The term "species" would then come to mean nothing more than a "described form". Fosberg (83) found no evidence that taxonomists in general incline to Uittien's views.

Stout (201) discusses the nomenclature of cultivated plants only, but his remarks contain matter of interest to the student of wild hybrids.

ANALYSIS OF POPULATIONS

Anderson (7) evolved a method of analysing hybrid populations in *Tradescantia* that, it is claimed, diagrammatically reveals their construction. The plants were scored by pairs of contrasting characters, each character being assigned a numerical value. The not altogether happy term "mass collection" was introduced by the same author (10, 11) for the critical sampling of entire populations. Anderson and Hubricht (12, 13), by analytical methods, studied "introgressive hybridization; the infiltration of the germplasm of one species into that of another by repeated back-crossing of the original hybrids". Only slight morphological effects were noted in the cases studied. Confirmatory hybrids were secured in the experiment garden. Riley (166–168), by similar methods, studied colonies of *Tradescantia* and *Iris*. In the *Tradescantia* colony a character analysis was made of 52 plants, the entire region being inhabited by three species. Few of the plants corresponded in every detail to any of the species. Crossing was common between the two tetraploid species, but uncommon between the diploid and the tetraploids. The triploid progeny were highly sterile. Evidence of introgressive hybridization in *Tradescantia* was also secured. A comparative study of seven characters on plants of four populations of *Iris* showed that one was pure *Iris hexagona* var. *giganticaerulea,* one pure *I. fulva*. Two colonies, situated geographically between the two pure species, in an area disturbed by man, showed the presence of hybrids that had become well established. "Many of the types considered species by Small show the same relation to the two

species . . . that the hybrids of this study exhibit, and are probably themselves hybrids". These methods show a distinct advance, but do not gain complete objectivity; there is still room for unconscious bias in weighting the index values.

Anderson and Turrill (15) made detailed studies on two populations of *Fraxinus,* from collections representing random samples, one from the Danube delta, and one from the Mesta delta. The data suggest "that two species are involved, and that though much mixing has taken place since the two met they still tend to maintain their entities". The two hybrid populations agreed in their main features, but differed in the degree of approach to the two species concerned. Marsden-Jones and Turrill (137) analyzed samples of *Silene* populations from Norway and Switzerland, showing "peculiar combinations of characters which, it is suggested, may represent ancestral combinations from which *S. maritima* and more typical *S. vulgaris* have been derived". Charles and Goodwin (36) used analytical methods in establishing that the intergrades between two species of *Solidago* were natural hybrids. Larisey (132) analyzed a hybrid complex between two species of *Baptisia,* finding a considerable measure of poor pollen.

Fassett (66–70) studied by the mass collection method populations of *Rubus, Ranunculus, Diervillea, Lonicera, Oxalis* and *Linaria.* Analytical methods were also used in various papers cited elsewhere in this article.

GENETICAL AND CYTOLOGICAL STUDIES

Allan (1) showed the remarkable differences in fruit colour in the F_2 of a cross between two species of *Coprosma* (female grandparent blue; male, red) ranging from milk-white through yellow to scarlet, orange and red, with blue spotting more or less developed in many, but blue as the main colour in very few. A similar but not so extensive range of colour is seen in wild populations. Anderson (8) gave a review of cytology in relation to taxonomy, including the significance of amphidiploidy. Anderson (9), under the term "binary variation", shows that associated with typical *Tradescantia bracteata* throughout its range, and connected with it by a manifold series of intermediates, is a form of *Tradescantia* morphologically similar to *T. hirsutiflora.* This variant may have originated by partial differentiation from or hybridization with the

T. hirsutiflora-virginiana stock at a time when the ranges of these species were confluent. Anderson and Sax (14) deal with cytological aspects of the *Tradescantia* work and give further evidence on introgression.

Babcock and Stebbins (19) show that in *Crepis* many autopolyploids are as apomictic as allopolyploids. Baldwin (23) concludes that *Sedum pulchellum* is an amphidiploid that has undergone autopolyploidy. Certain resulting taxonomic confusions are pointed out. Charles and Goodwin (36) estimate that a minimum of 21 genes for leaf-characters, and twice as many for all morphologically distinguishable differences, is required to differentiate two species of *Solidago*. Clausen (38) studied polyploidy in *Nicotiana* and gives a résumé of the position. He considers that there is abundant evidence that *N. rustica* and *N. tabacum* are amphidiploids, and that the relationship to South American species is well established. Darrow and Camp (60) show that in *Vaccinium* and related groups both autopolyploidy and allopolyploidy have taken part in producing polyploid populations. A complex phyletic pattern has developed. Dillewijn (62) found strong cytological evidence for the existence of hybridism in *Populus*. Flovik (79), discussing the vexed question of the origin of hereditary vivipary in grasses, gives evidence that allopolyploidy is a cause in various species. The viviparous arctic grasses are allopolyploids, and vivipary is more common in arctic than in other grasses, owing to combined action of hybridization, polyploidy and extreme external conditions.

Fothergill (84, 85) examined wild populations of *Viola lutea* and *V. tricolor,* and made progeny tests. Many combinations of the parental characters occur, but plants not intermediate tend to resemble *V. lutea* more than *V. tricolor*. The hybrids showed increase of chromosome numbers beyond that expected from those of the parents, apparently owing to the splitting of univalents. Neither of the original species was recovered in its entirety. Frankel and Hair (87) found that *Hebe* has basic numbers 20 and 21, and includes tetraploid and hexaploid species. Interspecific crosses were completely successful only between 20-chromosome species; other species crosses gave rise to progeny of low vitality. Goodspeed (94) gives a full review of work on the cytotaxonomy of *Nicotiana*. He concludes that "The evidence of phylogenetic relationship in *Nicotiana* which is intrinsic in the morphological and distributional

data appears to be sufficiently confirmed and amplified by the cytological conclusions to justify the current taxonomic arrangement of the genus".

Goodwin (95–98) confirmed the reputed hybridity of *Solidago asperula* by artificial hybridization. Hybrids and back-crosses occur in abundance where *S. rugosa* of open meadows is able to meet *S. sempervirens* of salt marshes. A morphological analysis of leaf-characters by Anderson's method (7) indicated that back-crosses and perhaps F_2 and other more complicated crosses frequently occur. In a study of seedling development it was found that the F_1 hybrids show a very strong matroclinous tendency. Greenleaf (101–103) found that cytological studies support the view that *Nicotiana tabacum* arose as an amphidiploid from *N. sylvestris* and some one of the several races of *N. tomentosum*, or *N. tomentosiformis*. This occurred in the general region of South America (Peru, Bolivia, northern Argentina) where these species and races occur to-day. Hair (110) found no irregularities in the meiosis of 17 species of *Epilobium* and three possibly hybrid forms. Heilborn (111) studied the chromosomes of Cyperaceae and concluded that allopolyploidy seems to be lacking in the family. B. L. Johnson (121), working on *Oryzopsis*, found that the section Piptatherum and the genus *Stipa* in part represent divergent lines of specialization and may have reticulated to form the polyploid section Eriocoma. With Rogler (122) he adduced strong cytological evidence for an *Oryzopsis* × *Stipa* hybrid. L. P. V. Johnson (123) noted some features of F_1 and F_2 hybrids in a *Populus* cross.

Kostoff (126–128), continuing his studies on polyploids in *Nicotiana*, synthesised a fertile amphidiploid by crossing *N. sylvestris* and *N. tomentosiformis*. He discussed numerous inter-specific crosses, several giving rise to amphidiploids. Müntzing (144) argues that "it is typical of species hybrids that they are more or less sterile", and that "by crossing experiments it is possible to clear up the barriers of incompatibility and sterility occurring in nature". He concludes that "If the cross succeeds without difficulty, and the hybrids obtained are quite fertile, the parents must be closely related and must be included in the same species". If this were followed by a New Zealand taxonomist the evidence at present available would strongly suggest that the manifold forms of *Coriaria* should again be united under one name, as was suggested by Lindsay in

1868 (Contributions to New Zealand Botany). In *Hebe* one would have largely to go back to the views of Mueller (The vegetation of the Chatham-Islands) who in 1864 lumped many species under his all embracing name of *Veronica forsteri.* Danser's method seems distinctly preferable for such cases.

Müntzing (145), continuing his studies on apomixis and sexuality in *Poa,* finds that "the evidence now available strongly suggests that the viviparous types are differentiation products, arisen by mutations from non-viviparous strains". He remarks, however, that "the possibility must not be overlooked that the whole species complex, *Poa alpina,* might be of hybrid origin". Nannfeldt (149, 153) gives evidence that the viviparous *Poa jemtlandica* is a hybrid and that the present population "is of rather high age, descending from a limited number of successful crossings, perhaps from a single one". Certain sterile hybrids in *Poa* show no trace of vivipary. Others, possessing viviparous biotypes, form both viviparous and non-viviparous hybrids. It is concluded from a survey of the evidence that "vivipary in all European and Arctic hybrids is explained by the hypothesis of vivipary as a hereditary dominant character. . . . No facts support the view that vivipary is induced in hybrids as a compensation for their floral sterility". The section Stoloniferae of *Poa* is thought probably to have arisen from intersectional hybridization. It contains no uniform species; the constant units are non-sexual subspecies.

Östergren (158) studied the cytology of *Agropyron junceum, A. repens* and their wild hybrids. Seven hybrids were found to be pentaploid and one heptaploid by back-crossing of a pentaploid with *A. junceum,* or from the fertilisation of an unreduced gamete of *A. junceum* with a normal one from *A. repens.* Silow (178) brings out evidence in support of Skovsted's view that the New World tetraploid cultivated cottons arose by amphidiploidy following hybridization between ancestral Asiatic and American diploid species. Smith (181) gives evidence from various sources that long continued isolation does not necessarily result in cross-incompatibility, nor does great morphological divergence. Stebbins (186), after a review of the data concerning apomixis in the angiosperms, finds that the great majority of apomictic forms are probably allopolyploids or back-cross derivatives from allopolyploids. The hybridity is an accompanying phenomenon but not a

causal agent of apomixis. Stebbins (189) reviewed also work on the cytological analysis of species hybrids. The evidence is considered to support the hypothesis that "speciation comes about either through hybridization between pre-existing species or through superposition of genetic isolating factors upon a pattern of intraspecific differentiation into races or subspecies, as postulated by Mayr". Stebbins and his co-workers (192–194) found that all but a few of the *Crepis* apomicts are allopolyploids, that *Bromus carinatus* and its relatives form a complex of polyploids (many being allopolyploids), and that *B. arizonicus* is an allopolyploid derived from *B. catharticus,* or an ancestral form, and *B. trinii.* Wetmore and Delisle (209) confirmed that *Aster amethystina* is a natural hybrid. *A. multiflorus* and *A. novae-angliae* hybridize extensively in nature, with back-crossing, producing a polymorphic group, to forms of which have been given different names by taxonomists. Wheeler (210), from cytological examination of 15 species of *Nicotiana* found in Australia and the Southern Pacific, and of the meiotic behaviour of numerous hybrids, suggests that amphidiploidy has been important in the evolution of the group, together with selfing or back-crossing after former hybridization.

ECOLOGICAL SIGNIFICANCE

As early as 1923 Cockayne (50), discussing hybridism in the New Zealand flora, remarked: "It is the fact of considerable hybridization taking place in a *virgin* vegetation which is the leading motive for this paper. But the *effect of settlement* is also an important matter. . . . In these man-induced associations a few species have found more opportunities for crossing than formerly so that their hybrids are now far more numerous than in primeval New Zealand. To the powerful effect of man in unconsciously favouring hybridisation Professor Aug. Henry has recently called my attention". Instances are given in the genera *Acaena, Cassinia, Celmisia* and *Fuchsia.* A particular *Nothofagus* forest had been replaced by a subalpine vegetation in which *Celmisia* hybrids were prominent at a much lower altitude than their normal habitats. Cockayne, Simpson and Thompson (53), describing new communities that had arisen following the destruction of the primitive vegetation by man, referred to the occurrence of hybrid groups in *Acaena, Aristotelia, Cassinia, Celmisia, Coriaria, Gaultheria, Hebe, Helichrysum*

and *Myrtus*. Cockayne and Sledge (52) mention several hybrids as being present in a particular area where there had developed a herbfield replacing burnt forest.

Weigand (211), studying occurrences of *Amelanchier, Crataegus* and *Rubus,* found that an outburst of crossing may occur locally in areas in which the primitive vegetation has been disturbed, *e.g.,* by forest fires, lumbering or clearing of railroad tracks. One result of the disturbance may be irregularities in the time of flowering. "The hybrids seem like swarms of bees, buzzing around for a time, only to disappear, leaving the fundamental species to continue through the ages". He discusses genetic reasons for this disappearance and considers that taxonomists tend to collect undue proportions of unusual forms. The importance of the disturbance of natural vegetation and the production of intermediate habitats is also stressed for *Aster* (11), *Tradescantia* (12), *Solidago* (36, 96) and *Iris* and *Tradescantia* (167, 168).

Hubricht and Anderson (115) state that certain species of *Tradescantia* readily hybridize by natural agencies in the experiment garden, but that in nature, owing to lack of intermediate habitats, hybridism is rare. Hybridization is more active along the Appalachians, as more intermediate zones are found there than elsewhere. Darrow and Camp (61) adduce evidence that the present prevailing hybridism in *Vaccinium* has been made possible by "the disturbance of the ecological balance—the extensive and repeated clearing and burning, the formation of fields and pastures, and often their later abandonment". Bees are considered to be the agents of pollination. "In the blueberry there seems to be no interspecific sterility between homoploids". Each species seems to have a basic genom set which is compatible with that in other species. Because self-sterility occurs to a greater or lesser extent in the genus, the development of species seems to have been determined more by geographical and ecological isolation than by genetic isolation. Smith (182) states that artificial hybrids have been produced in abundance in *Populus,* and the comparative scarcity of natural hybrids is attributed to the different ecological habitats favoured by the different species. Stebbins (187) discusses the general question of the rôle of isolation in the differentiation of plant species. Turrill (203) points out that *Alopecurus geniculatus* and *A. pratensis* will hybridize but are kept "essentially distinct" by

their different habitatal preferences. Several similar cases are referred to. He concludes, "There is, however, no doubt that man is likely to become increasingly important in modifying nature by enabling new species to establish themselves. Thus by cutting down forests on the hill-slopes in the Nearer East species of *Verbascum, Thymus, Dianthus, Silene,* and other genera have been able to extend their areas, to meet, and to hybridize. That certain segregates or allopolyploids resulting from such hybridization may be selected or are in process of selection seems probable". Heine (113) thought that "The similarity and simplicity in the structure of the flowers and the great extent to which cross-pollination is brought about by a variety of insects may be regarded as one of the causes of the large number of wild hybrids in New Zealand". Cain (29) points out that "Such centers of variation as are due to hybridization and polyploidy may develop at the center of origin of a genus, but that is not necessarily the case". The same author (30), in a general treatment of the principles of plant geography, gives a great deal of space to the influence of wild hybridism, with a wide range of illustrative material, whereas Wulff (212), treating plant geography from the historical point of view, finds little occasion to refer to wild hybridism.

EVOLUTIONARY SIGNIFICANCE

The importance and limitation of polyploidy, more especially allopolyploidy, as a factor in evolution have been increasingly explored, with considerable reference to wild allopolyploids. The phenomena are discussed in some detail in most recent standard texts (*e.g.,* 30, 63, 93, 117). Huxley (117) sums up, "Allopolyploidy has undoubtedly played an important rôle in the evolution of many plant genera". Allopolyploidy "is likely to occur when changes of climate bring about range-changes, these then providing opportunities for hybridisation between plant species which have developed in isolation and between which no reproductive barriers have therefore been evolved". Huxley also discusses the bearing of wild hybridism in general on the evolution of plants, and its relation to apomixis and "reticulate evolution". Where "some of the products of initial hybridization continue to cross, we obtain elaborate complexes such as those of *Rosa, Rubus, etc.,* in which a number of initial forms are combined in an evolutionary reticulum".

Stebbins (185) agrees that polyploidy tends to break down genetic barriers and to permit exchange of genes between genetic systems that in the diploid condition are completely isolated. He gives examples to show that where autopolyploidy and allopolyploidy exist in the same genus hybridization, especially when associated with apomixis, may result in a "complex network of interrelated forms, which defies classification according to the usual concepts of the species". Warmke (207) suggests that polyploidy may offer a means of securing new genes required in the building of plant and animal kingdoms. Babcock and his co-workers (17–19, 21) discuss genetic evolutionary processes in *Crepis*. Gene mutations and structural changes in the chromosomes are primary, and interspecific hybridization, polyploidy and apomixis are secondary processes. The main trend has been a progressive decrease in chromosome numbers, but in special groups there has been an increase due to hybridization followed by amphidiploidy.

Barber (25) finds that the two species of *Paeonia* in California and Oregon are "true-breeding diploid hybrids of the kind known in *Oenothera*". Babcock *et al.* (20) find that the American species of *Lactuca* are amphidiploids that have probably crowded out the ancestral diploids. Erlanson (65) considers that in *Rosa* the hybridization of hexaploids with related diploids, followed by the elimination of unpaired chromosomes in the descendants, is "an important *modus operandi* in promoting the appearance of polymorphic diploid races with novel characteristics". Lamprecht (131) studied the nature of the interspecific barriers in a *Phaseolus* cross, where in the sterile hybrid mitosis was normal, but meiosis did not proceed beyond tetrad formation. Some evolutionary significance is ascribed to the types of barriers discussed.

Ford (80, 81) distinguishes transient polymorphism, in which a gene is spreading through a population, from balanced, in which the gene is maintained at a fixed level by opposed selective agencies. Under certain conditions the free flow of genes is impeded, evolutionary change reduces the stock of genes, "leading to infertility on crossing and to specific distinction".

PRACTICAL APPLICATIONS

Following the demonstration by Allan and Zotov (5) that *Phormium colensoi* could be crossed with *P. tenax*, supporting the

view that the polymorphy noted in nature is due in part to hybridism, Swindlehurst (unpublished) raised a large F_1 progeny, the plants combining in some degree the strength of the one species with the softness of fibre of the other. Tests have shown that the cross offers distinct possibilities in the textile industry. Crane (54) gives examples of wild hybrids that have proved useful in the production of forms of horticultural value. Darrow and Camp (61) show how natural *Vaccinium* hybrids may be used in securing new material of economic value. Wild forms of *Leptospermum* in New Zealand have been introduced into gardens on account of specially attractive features of flower colour and "doubling". Lammerts (130) successfully crossed certain horticultural "varieties" and secured strains of enhanced merit. He discusses the genetic problems raised.

Love (135) points out that natural interspecific hybridization in *Stipa* may be utilized in developing useful strains. Stebbins (190) points out the practical significance of hybridization in *Bromus*. Weibel and Woodworth (208) discuss the use of the "natural-crossing plot" in making castor-bean hybrids. In general, it is clear that there is a wide field for profitable investigation on the practical application of the data arrived at in the study of wild hybrids.

POSTSCRIPT

Baker (Nature **159**: 221–223), under the title "Criteria of hybridity", deplores the "decline" of experiment since Kerner's day. He gives a useful criticism of the methods of Anderson and others, using the index method of scoring hybrids, in that they too greatly depend upon the observer's powers of discrimination. He says that "It is easy to overcome the faults in these methods by making it a rule that, wherever possible, artificial crosses between the forms involved (giving evidence of the mode of inheritance of the characters) should *precede* investigations of natural populations". He realises some of the difficulties in carrying out such work and goes on to consider how the most suitable characters can be selected and used in analytical work.

There has of course been experimental work on the lines Baker advocates, but it can hardly be denied that much more work should be done. The results of his own work will be awaited with interest. Baker makes the encouraging, if bold, statement that "The impor-

tance of the study of natural hybridization is obvious in view of the large part it plays in the process of evolution".

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CYTOLOGY OF BACTERIA. II[1]

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INTRODUCTION

Since the author wrote (1938), in this journal, his first review on the cytology of bacteria, interest in the structure of the bacterial cell and knowledge of that structure have considerably increased. This was due to a number of reasons: development of the electron microscope; application of new techniques and refinement in old ones; development of new points of view. It would be presumptuous to say that present knowledge of the structure of the bacterial cell is complete. It is certain, however, that the progress of the last decade transformed bacterial cytology from a philosophy into a science in which structure and behavior are beginning to show relationships and common grounds. This, indeed, is the "raison d'être" of cytology.

Books and reviews published during the last ten years were the following: Imšenecki (1940), Lewis (1941), Knaysi (1944), Mudd and Anderson (1944), and Dubos (1945). This brief review will be devoted, chiefly, to the progress made during this period. The literature is covered till the closing months of 1947.

FORM AND SIZE OF THE BACTERIAL CELL

Form

There are three fundamental forms which can be assumed by a normal bacterial cell: the coccus form in which the cell is ellipsoidal or spherical; the rod form in which the cell is cylindrical or resembles a long ellipsoid; and the spiral form in which the cell is helicoidal. The terms "spherical", "ellipsoidal", "cylindrical" or "helicoidal" are descriptive of what approaches the corresponding geometrical form.

In a young or a mature culture growing under uniform conditions, a given form may predominate, but almost always deviation from that form may be observed. In a culture of *Streptococcus*, for instance, rod-like forms may often be observed; a culture of a rod-like

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strain may contain both coccus and helicoidal cells; cultures of *Vibrio* or *Spirillum* may contain coccus and rod-like cells. In old cultures the mixture of forms becomes more common, and many cells assume irregular or involution forms. Certain strains, called "pleomorphic", regularly exhibit mixtures of forms, even in young cultures. Variation of form in a uniform environment may be due to irregularities in cell division, as in a mixture of coccus and rod-like forms, or to local irregularities in the cell wall, as in a mixture of rod-like, wavy and helicoidal forms. In cells grown on the surface of agar, the shape of a cell may be altered by friction or other physical factors. Recent observations by Pijper (1946, 1947*a*) on *Eberthella typhosa* show that motility induces a helicoidal form which persists after motility ceases. Pijper (1946) states that "the conventional description of bacteria as rods is due to their appearance in killed, fixed and stained preparations. . .". Since the normal shape should be that characteristic of the active state, "the normal shape of a motile bacterium is not rod-like but is that of a spiral or a helix. To how many bacterial species this conception applies is difficult to say at the moment. It does not seem excluded that even those that are non-motile nowadays still preserve an indication of a previous more active period and shape." This view is opposite to that previously expressed by Ellis (1923) who considered *Spirillum* as a rod-like organism with a delicate membrane, which assumes a helicodial form because of its motion.

The form of a cell determines the relation between its surface area and its volume, and thus affects its relation to environment. The most valuable expression of form is the one which relates most closely the form of the cell to its volume and its surface area. In deriving such an expression, strictly geometrical form is assumed which in many instances is not too far from the truth. Expressions suitable for coccus and helicoidal cells were applied and discussed by Knaysi (1941*b*, 1944). No similar expression has yet been derived for rod-like cells, and the ratio between width and length is still used for this purpose. The mathematical study of form and its value may be illustrated by the work of Henrici (1928) and Knaysi (1941*b*).

Size and Other Dimensional Relations

Knaysi (1944) published formulae for the rectification of helicoidal and wavy dimensions as well as for the estimation of volumes and surface areas of cells for all three standard forms.

Experimental Determination of Form and Size

Almost always the form and size of the bacterial cell are determined directly by means of the microscope. The direct methods commonly used may be classified as follows:

1. With the electron microscope.
2. With the light microscope:
   a) on living cells
   b) on stained cells
   c) on dried, "negatively stained" cells.

These various methods will now be discussed and evaluated.

With the Electron Microscope. Dubin and Sharp (1944) observed that "The appearance of *Bacillus megatherium* in electron micrographs is different from that of the same organism seen in light micrographs. Images in the electron micrographs indicate plainly the presence of two structures constituting the bacterial cell, an inner dense substance and an outer less dense substance. This outer substance of the bacterial cell is invisible in the light micrographs. . . . There is no significant difference between measurements of the size of unstained bacteria dried in air and photographed with the light microscope and measurements of the comparable portion (inner dense substance) of the identical bacteria later photographed in the electron microscope." A glance at the illustrations published by Dubin and Sharp shows that the "outer substance" which is invisible with the light microscope is the cell wall which surrounds the "inner dense substance of shrunken protoplasm."

Recent work by Hillier and Knaysi (1947*b*) showed that when a bacterial cell is dried in the air on a collodion film it becomes flattened and its protoplasm shrinks to a variable degree; the cell wall also shrinks but usually much less than the protoplasm. Collapse of the cell wall tends to increase the width of the cell in relation to its length and thus affects its form. However, this latter effect is more than counterbalanced by the shrinkage of the cell wall, so that cell width in electron micrographs is smaller than in tne living cell.

With the Light Microscope: a) On the living cell. When a bacterial cell is observed in the living state, one observes only the protoplasm surrounded by the cytoplasmic membrane. The cell wall is not visible. Consequently, the cell appears slightly more slender and smaller than it is. Because of the thinness of the cell wall, however, the form and size observed by this method are nearest

to the true form and size of the cell. This statement is true only when the cell is observed in its own culture medium. Transfer of the cell into a medium of different osmotic concentrations usually results in swelling or shrinkage of the cell. Dutky (1933) observed a linear cytoplasmic contraction of 14% to 25% when young cells of *Bacillus megatherium* were transferred from ordinary broth to broth containing NaCl in 2-molar concentration, and Lasseur, Dupaix, and Georges (1932) observed an increase in volume from 0.100 to 0.140 μ^3 when cells of *Pseudomonas caryocyanea* were transferred from physiological salt solution to distilled water.

b) *On the stained cell.* Until recently form and size were studied in dry, stained smears, but it was shown by Knaysi (1941*a*) that what one sees in such smears is a shrunken protoplasm which has neither the form nor the size of the living cell. On the other hand, when the bacteria are stained in a way that renders the cell wall visible, and observation is made on wet smears, the form and size of a cell are not significantly different from its form and size in the living state.

c) *On dried, "negatively stained" cells.* This has been a very popular method with certain investigators. Dubin and Sharp (1944) found that "Measurements of the size of *B. megatherium* obtained by Benians' Congo red method were less than those of the total bacterial substance seen in the electron micrographs." Knaysi (1944) pointed out that "Dry negative preparations are often unsuitable for the study of form, particularly in capsulated organisms, because the slime layer is included in the apparent outline of the cell." Capsulated organisms also appear considerably larger. Using *Bacillus mycoides*, Knaysi (1945*b*) showed that in the absence of capsules "the cells may appear larger or smaller than their real size depending on the thickness of the dye film surrounding the cells."

Variability of Form and Size

Since the bacterial cell is in direct contact with the environment, its form, size, metabolic activities and other characteristics are greatly affected by the environment. These aspects were adequately discussed by Knaysi (1944) who also pointed out that, at least in bacteria, what is usually attributed to age is the result of the process of growth or of alteration in the environment brought about by the activities of the bacteria themselves. Changes due to age were called "cytomorphosis" by Henrici (1928).

a. Imposed Environment. It was shown by Knaysi (1943*b*) that the effect of $MgSO_4$ on cell morphology, previously observed by Stapp and Zycha (1931) in *Bacillus mycoides*, may also be observed in other bacteria. In *Escherchia coli* $MgSO_4$ causes enlargement and deformation of the cells. Besides yeast-like cells, one observes ring-like forms and numerous enlarged curved cells often with pointed ends. The cytoplasm is highly vacuolated. This effect was correlated with osmotic pressure and permeability. Bayne-Jones and Sandholzer (1933) observed enlargement or swelling of the cells of *Escherchia coli* before lysis with the bacteriophage. Some of the enlargement was an expression of increase in cell substance, and was not altogether due to imbibition of water. This was not observed in *Bacillus megatherium.*

Most of the literature which appeared on this subject during the last ten years dealt with the effect of sulfa drugs (Gay and Clark, 1937; Lockwood, 1938; Tunnicliff, 1939), of penicillin (Gardner, 1940; Fleming, 1941; Smith and Hay, 1942; Hobby, Meyer, and Chaffee, 1942; Weiss, 1943; Miller and Foster, 1944; Miller, Scott and Moeller, 1944; Hobby and Dawson, 1944; Lee, Foley, and Epstein, 1944; Rantz and Kirby, 1944; Todd, 1945; Chain and Duthie, 1945; Thomas and Levine, 1945; Alture-Werber, Lipscitz, Kashdan, and Rosenblatt, 1945; Fisher, 1946; Shanahan, Eisenstark, and Tanner, 1947; Pratt and Dufrenoy, 1947; Dufrenoy and Pratt, 1947) and of tyrothricin (Johnson, 1944). The effect is expressed by enlargement and deformation of the cells, lysis and, in Gram-positive bacteria, often an alteration in this reaction. It is interesting that in pneumococcus the capsule persists after the protoplasm is lysed. The effect was found maximum during the active growth of the culture and was attributed by most investigators to interference with the mechanism of cell division. On the other hand, Garrod (1945) found the maximum effect at 42° C. after growth had stopped.

b. Age. Variation of form and size in a given medium during the early stages of development are largely due to growth and multiplication. The work of Clark and Ruehl (1919) and of Henrici (1928) on this subject was previously reviewed (Knaysi, 1938, 1944).

A cinematographic study of the growth of cell volume with time during active growth was made by Bayne-Jones and Adolph (1932*a*,

1932*b*) on the yeast *Saccharomyces cerevisiae* and on *Escherichia coli*, and by Adolph and Bayne-Jones (1932) on *Bacillus megatherium*. When the volume of a single yeast cell is plotted against time, an S-like curve is obtained; plotting the logarithms of the volume against time gives a curve which is continuously concave toward the time axis. In the case of *E. coli*, logarithms of the volume plotted against time give a wavy curve which, as the culture becomes older, tends to become irregular but seems to approach an S-shape. With *Bacillus megatherium* volume against time gives a curve continuously concave toward the volume axis, and the logarithms of the volume against time give a straight line. Adult lengths, breadths and volumes of the bacteria "were progressively modified at various ages of the culture. Mean sizes and total bulk in the culture appeared to depend upon rates of growth and of reproduction. Evidence was adduced that rates of growth controlled rates of reproduction. Adult sizes could be considered more primary than rates of reproduction in controlling the characteristics of individuals." Perhaps the most interesting aspect of this work is the finding that growth of the yeast cell ceases and resumes with the formation of each new bud, a relation not found between consecutive generations of the bacteria they investigated. This work was followed up by Knaysi (1940) who found that the rythmic relation in growth is also present in the dividing yeast *Schizosaccharomyces pombe* but not in *Bacillus cereus*. Consequently, rythmic growth is not the result of multiplication by budding. In a later study Knaysi (1941*b*) observed rythmic growth in *Streptococcus faecalis* and suggested that it may be a characteristic of uninuclear cells. The "resting" cell of this organism has the form of a slightly flattened ellipsoid; when transferred to a fresh medium it gradually elongates into a sphere and then into an elongated ellipsoid; when the volume reaches a maximum of 2.7–3.4 μ^3 the cell divides into two cells each having the shape of a flattened ellipsoid (E about –0.5). The width of the cell undergoes only a very slight change during the process. In *Bacillus cereus* there is no definite volume at which the cell divides. Knaysi also found that the cell of *Streptococcus faecalis* divides when the ratio of surface area to volume reaches a certain value (3.6–3.8). In *Bacillus cereus* this ratio is practically constant. When the entire culture of *Streptococcus faecalis* is considered, the predominating form and size are

determined by the statistical condition of the culture with respect to form and dimensions of its cells, which is determined by the statistical conditions of the cells with respect to growth and multiplication. In young cultures the average cell volume fluctuates considerably with time and becomes more steady as the culture matures. The average form follows a somewhat parallel curve.

Dissociation

Inheritable changes in form and size may be caused by dissociation. It must be emphasized, however, that the resulting strains are affected by age and environment in the manner described above.

The literature on variations of this type is extensive and was recently reviewed by Braun (1947) and by Luria (1947). A characteristic tendency of this period has been to consider these changes chiefly mutational, a point of view which is justified by the similarity of organization between the cell of bacteria and that of other organisms. It is hoped, however, that the geneticist will show discrimination in applying knowledge gained from the study of higher organisms to bacteria.

STRUCTURE OF THE BACTERIAL CELL

During the last decade considerable progress was made in knowledge of the structural make up of the bacterial cell. The existence of a cytoplasmic membrane and a cell wall was definitely proved. A nucleus was demonstrated in a few species, and there are reasons to expect that its existence is more general than heretofore believed. The structure of the endospore was considerably clarified. The flagella became again a subject for investigation. Furthermore, much was learned about some important cellular processes, such as multiplication and spore formation. Notable advances were made in knowledge of the chemical and antigenic composition of various structures, and in certain instances their behavior was related to certain cell properties and cellular processes.

Cytoplasm and Cytoplasmic Membrane

The cytoplasm is that ground material which, together with the surrounding cytoplasmic membrane, occupies the cavity of the cell wall. When free of inclusions the cytoplasm is optically homogeneous and appears so even with the electron microscope. It is

probably a colloidal system containing water, protein, lipoids, usually ribonucleic acid and other substances in which the relation to one another is uncertain. In *Spirillum volutans* the viscosity of the cytoplasm is not unusually high as compared to that of organisms other than bacteria (King and Beams, 1942).

The cytoplasmic membrane is a highly refractive and stainable structure of variable thickness. In the living cells of *Bacillus cereus* it varies in thickness from 0.2 to 0.3 μ (Knaysi, 1946a). In electron micrographs the cytoplasmic membrane of *Bacillus mycoides* appears very thin. The difference is probably due not only to a difference between organisms and to desiccation, but also to the fact that the thickness observed with the light microscope is magnified by a diffraction halo.

The cytoplasmic membrane normally stains with dyes of the Sudan series, gives the Sharp test for proteins, and a positive Feulgen reaction. These characteristics survive autolysis of the cytoplasm and treatment with fat solvents; it has an isoelectric range below that of simple proteins, even in Gram-negative species (Knaysi, 1946*a*). These reactions lead to the conclusion that the principal component of the cytoplasmic membrane is a stable combination of lipoids and proteins, namely, lipoprotein. There is also evidence that this lipoprotein molecule may be conjugated with some complex organic radical related to ribonucleic acid. It should be emphasized, however, that the composition of the cytoplasmic membrane is by no means constant; its lipoid and nucleic acid content varies considerably. The work of Knaysi and Baker (1947*b*) with the electron microscope showed that the membrane of *Bacillus mycoides* consists of molecules 40 Å to 60 Å in width in the form of beaded threads.

In the life of the cell the cytoplasmic membrane is extremely important in cell permeability and cell division. In *Bacillus cereus* and *Bacillus mycoides* it is a site of lipoid synthesis (Knaysi, 1945*a*, 1946*a*).

The Nucleus

During the last ten-year period a number of contributions were made to the study of the nuclear problem. Delaporte (1939–40) concluded and extended her early work (1934, 1935, 1936*a*, and 1936*b*) on the structure of bacteria and the Cyanophyceae. Using a number of staining procedures and the Feulgen reaction, she re-

ported that in the cocci, chromatin is in the form of a granule; in rod-like bacteria, it is a granular rod which may break up into granules; in coccobacilli, such as *Azotobacter agilis*, it may be as in the cocci or as in rod-like cells; in *Spirillum* and the filamentous sulfur and iron bacteria, it is in the form of a central rod as in rod-like bacteria. Some of the red sulfur bacteria contain a central body; in *Chromatium* and *Achromatium* chromatin is either in the form of a central mass or fragmented into several portions. The paper includes an exhaustive review of the literature.

Stille (1937) reported that Feulgen-positive bodies may be readily observed in various bacteria if the temperature of hydrolysis is reduced to 40° C. Piekarski (1938, 1939*a*, 1939*b*, 1940) investigated the nucleus, using staining procedures, the Feulgen reaction and the ultra-violet and electron microscopes (see also Piekarski and Ruska, 1939). The results by all methods were similar: rod-like bacteria contain one or more nucleus-like bodies or nucleoids which divide before cell division; *Sarcina* contains a single central nucleoid. Young cells stained uniformly with acetic carmin or Giemsa's solution, but the nucleoids became readily demonstrable when the cells were stained after treatment with hydrochloric acid. Piekarski attributed the beneficial effect of HCl to loosening the structure of the cell membrane. Nucleoids were demonstrable with the electron microscope in individual cells from most old cultures of *Eberthella typhosa, Escherichia coli, Serratia marcescens, Proteus* and *Pseudomonas aeruginosa,* but rarely in cells of young cultures. In old cultures the cells contain a single central nucleoid; in young cultures there is a nucleoid at each end. Piekarski and Ruska (1939) confirmed these observations but found little structural differentiation in the cells of *Sarcina alba, Sarcina lutea* and *Bacillus subtilis.* Piekarski (1939*b*) was unable to observe structure in the nucleoid and concluded that it is not made up of individual elements (chromosomes). Chance (1938) and Allen, Appleby and Wolf (1939) observed nucleus-like bodies in members of *Bacillus.* Chance saw evidence of mitosis, and Allen *et al.* that of meiosis.

Knaysi (1941*c*, 1942) demonstrated in the cells of *Staphylococcus flavo-cyaneus* bodies which stained with methylene blue at pH 1.8–2.0, were insoluble in boiling water within ten minutes or in 0.02% $NaHCO_3$ within two hours, gave the Millon and the Sharp tests for protein and a positive Feulgen reaction. When the

smears were placed in a solution consisting of 90 ml. of distilled water and ten ml. of conc. HCl for 24 hrs. at room temperature, the bodies were not demonstrable with methylene blue at pH 2.4, but were still demonstrable at pH 4.6 to 4.8. They were distinct from any inclusions known to occur in the bacterial cell. Resting cells contained a single granule, round or slightly ellipsoidal; actively growing cells contained from two to four granules of shapes indicating elongation and division. Knaysi concluded that these bodies were, both chemically and morphologically, of nuclear nature.

Robinow (1942, 1944, 1945) combined staining with Giemsa's solution after fixation of the cell suspension with vapors of osmium tetroxide (Dobell, 1911) and hydrolysis by HCl (Piekarski, 1939*a*). He was thus able to demonstrate in vegetative cells or endospores of a number of bacteria bodies which in young cultures showed a possible evidence of division. These bodies were called "chromatinic structures" or, following Badian (1933, 1935), "chromosomes." Lewis (1942) showed that the important step in the process is that of hydrolysis; in cells treated with HCl these bodies may be clearly seen after staining with methylene blue. According to Robinow (1945) the effect of hydrolysis is chiefly to reduce stainability of the cytoplasm in vegetative cells and to increase permeability of the membrane in the endospores. Robinow was unable to observe the same bodies with the electron microscope.

Knaysi and Mudd (1943) studied the internal structure of a number of bacteria with the RCA electron microscope. Organisms opaque to the electrons at 60 kilovolts were also observed at higher voltages. At 60 kv, the young cells of *Staphylococcus flavo-cyaneus*, *Staphylococcus aureus* and *Streptococcus pyogenes* appeared homogeneously opaque to the electrons; those of *Neisseria gonorrhoeae* were homogeneous and semitransparent; those of *Brucella abortus* and *Pasteuralla pestis* showed a homogeneous transparent protoplasm containing one to several bright vacuoles. Old cells of the latter two organisms showed a transparent protoplasm containing one or two opaque bodies of unknown nature. Mature cells of *Neisseria meningitidis* had a transparent protoplasm containing one opaque body per cell; this body was insoluble in water at 80° C., indicating that it was not volutin. Treatment of the opaque cells with dilute sodium bicarbonate obscurely revealed the presence of internal bodies in *Staphylococcus flavo-cyaneus* but not in *Staphy-*

lococcus aureus. With the high-voltage microscope cells of *Staphylococcus flavo-cyaneus* from a two-day-old culture at room temperature were transparent and contained beautiful, often paired, opaque bodies which, liberated from the cells by sonic vibrations, gave a position Feulgen reaction and closely resembled the nuclei previously observed in this organism by Knaysi (1941*c*, 1942). Unpublished results of chemical analysis made by Smolens showed that these bodies were rich in phosphorus and contained purine nitrogen. Cells from young cultures, observed with the high-voltage microscope,were either less transparent with poorly defined nuclei or homogeneously opaque. The cells of *Streptococcus pyogenes* became transparent but showed no internal differentiation, although it was reported by Sevag, Smolens and Lackman (1940) that they contained relatively large quantities of thymonucleic acid. On the basis of these observations Knaysi and Mudd concluded that bacteria contain nuclear material which, depending on conditions yet unknown but probably related to environment and development, may be diffused in the cytoplasm or may be partly or totally differentiated into a nucleus.

This hypothesis appeared to have confirmation in the observation by Knaysi (1946*b*) that forespores and young endospores stain homogeneously by the Feulgen procedure, but that mature spores show a positive granule or rod. However, in the light of later work by the author the interpretation given to this phenomenon, which appeared obvious at the time, may not be the only one. It is possible that this observation is more related to the chemistry of the endospore than to its nuclear structure; for instance it may mean that the ribonucleic acid of the forespore and young endospore is not as readily removed by hydrolysis as the ribonucleic acid of the mature spore; ribonucleic acid may be free in the mature spore and in a stable chemical combination in the forespore and young endospore.

Baylor, Appleman, Sears and Clark (1945) studied the internal structure of *Rhizobium leguminosarum* with and without previous treatment with various solvents and nuclear stains. Observation was made with the electron microscope. Untreated cells and cells stained with Heidenhain's hematoxylin contained poorly visible, opaque bodies. Hydrolysis with HCl, application of the Feulgen technique or staining by several other procedures caused distor-

tion and increase in the opacity of the protoplasm. Only in cells treated with dilute sodium bicarbonate were the internal bodies clearly visible. These bodies were soluble in acetone and in 2% nitric acid. The authors concluded, however, that they may be nuclei. Nucleus-like bodies were observed in the colon-typhoid group by Peshkov (1945).

Bombardment of the cells of *Escherichia coli* with x-rays led Cavalli (1945) to conclude that the sensitive spot is smaller in old cells than in young ones, and that this explains the greater resistance of old cells.

Knaysi and Baker (1947) showed that the reason why the young cells of *Bacillus mycoides* usually stain uniformly and do not show internal differentiation when observed with the electron microscope is the presence of ribonucleic acid diffuse throughout the cytoplasm. Since the chemical composition and affinities of ribonucleic acid and of thymonucleic acid are similar, nuclei present in such an environment are masked, as would be a ball of glass immersed in a colorless liquid of equal refractive index. When the endospores of *Bacillus mycoides*, rich in ribonucleic acid, are placed in a medium containing a source of energy but no source of nitrogen, they germinate and grow, using their ribonucleic acid supply in the process. The vegetative cells which develop are transparent to the electrons, even at ordinary voltages, and each cell contains from one to six large opaque bodies which show evidence of division and play a leading rôle in the formation of the endospore. These bodies give the Sharp test for protein, a positive Fuelgen reaction and are distinct from any inclusions known to occur in the bacterial cell. They are demonstrable with methylene blue at a pH of 3.5 to 4.0, a property which they share with the nucleus of *Hansenula anomala* (Knaysi, 1946*d*). Although it was not possible to observe structural differentiation within these bodies, their microchemical reaction and particularly their behavior during the life cycle of *Bacillus mycoides* identify them as the nuclei of the cell. Knaysi and Baker presented evidence indicating that these nuclei do not divide by simple constriction.

For over half of a century cytologists have been observing in the cells of various bacteria bodies which, because of their tinctorial reactions or their occasional pairing or apparent constriction, were called nuclei. These criteria were critically examined by Knaysi

(1944) who pointed out that the only sure criterion for the nucleus is its behavior during the life cycle of the organism. The complexity of biological structures is such that no single chemical test or staining procedure can be relied on to determine the function of a structure. We are thus led to examine two procedures extensively used during the last decade, namely, the Feulgen reaction and the Piekarski-Robinow technique of staining with Giemsa's solution. In both procedures the cells are treated with acid, usually *n* HCl, for a given time at a given temperature. This treatment splits the purine bases from the thymonucleic acid, thus liberating aldehyde groups (Feulgen and Rossenbeck, 1924; Delaporte, 1939) and removes volutin or free ribonucleic acid (Knaysi, 1942, Figs. 16 and 17). A recent investigation by Vendrely and Lipardy (1946) shows that treatment with *n* HCl at 60° C. for ten minutes removes 37% of the desoxyribose nucleic acid from *Escherichia coli* and 7.5% of that substance from *Staphylococcus aureus*. It also removes most of the ribonucleic acid from both organisms. Since ribonucleic acid is responsible for the deep staining of the cytoplasm in yeasts (Knaysi, 1946*d*) and bacteria (Knaysi and Baker, 1947; Vendrely and Lipardy, 1946), its removal makes the cytoplasm poorly stainable, and unstainable at a moderately low pH. In the Feulgen reaction structures which are not removed by the acid treatment and which contain free aldehyde groups recolor Schiff's reagent. In the Piekarski-Robinow technique stainable structures which were not removed by the acid treatment become clearly demonstrable. In the bacterial cell it has been shown that the nucleus, the cytoplasmic membrane and its extensions, and certain presporal inclusions are not removed by hydrolysis and give a positive Feulgen reaction (Knaysi, 1944, 1945*a*, 1946*a*). Recent studies by the reviewer showed that the same structures also stain with Giemsa's solution, although more slowly than the nuclei. Confusion between nuclei and presporal granules has already resulted (Klieneberger-Nobel, 1945). There is also danger that bodies thus demonstrated may have been artifacts caused by coagulation and drying and wetting during the harsh manipulations to which the cells are subjected. For a first demonstration of a nucleus in bacteria the results of both methods could not be considered as a final proof, although both methods may be extremely useful when used discriminately on organisms of known nuclear structure. It

must be added that the fundamental process in the Piekarski-Robinow technique is the treatment with acid. Giemsa's solution has excellent tinctorial qualities, but it could easily be replaced by basic dyes such as methylene blue (Lewis, 1942). In cells which have little or no ribonucleic acid, such as the transparent cells of Knaysi and Baker, the nuclei may be readily demonstrated by a modification of Burke's Gram-staining; the nuclei appear as Gram-positive bodies in a Gram-negative cell. Recently Lamanna (1947) observed and photographed nucleus-like bodies in germinating spores of *Bacillus cereus* stained by Burke's Gram-staining method, an unreported observation he had made earlier (1941) on *Bacillus subtilis*. During germination and for a few generations afterwards the ribonucleic acid content of the cell is considerably reduced (Dyar and Knaysi, 1947).

Since nucleus is a morphological concept, the nuclear nature of a body can be proved only by its behavior during the life cycle of an organism, a condition fulfilled only in the work of Knaysi (1942) with *Staphylococcus flavo-cyaneus* and in that of Knaysi and Baker (1947) with *Bacillus mycoides*. In the first case it was shown in addition that the bodies observed consisted of the nucleoprotein characteristic of the nucleus. In the second case the bodies observed had several of the microchemical and tinctorial properties of known nuclei. The fact that these bodies have not yet shown a chromosomal structure should not be given weight against their nuclear nature and their nuclear behavior: chromosomes may be present but unresolvable with the light microscope; chromosomes may be present but, because of the thickness and density of the bodies, they are not distinguishable with the electron microscope; finally, each body may consist of a single chromosome as assumed by Badian (1933, 1935) and by Robinow (1945).

Consequently a nucleus has been satisfactorily demonstrated in the cell of *Staphylococcus flavo-cyaneus* and in the cell of *Bacillus mycoides:* it will probably be demonstrated in other bacteria. On the other hand, it is not advisable to assume that all bacteria are similarly constituted until representative of all groups of bacteria are reinvestigated.

Cell Wall and Slime Layer

The Cell Wall. Although the existence of a cell wall in bacteria was demonstrated by Fischer (1891), this either was not generally

known or was not generally accepted by bacteriologists. While studying the process of cell division, Knaysi (1941*a*) developed a simple method by which the cell wall can be clearly seen: the dried cell smear is fixed by heat, mordanted with a mixture of potassium alum and tannic acid, and examined under a film of carbol fuchsin. The cell wall appears blue, the cytoplasmic membrane dark red, and the protoplasm a lighter red. The cell wall is readily observed with the electron microscope and shows evidence of considerable ductility; when the cells are broken by sonic vibrations, the fragments of the cell wall appear jagged, indicating a certain degree of rigidity (Mudd, Polevitzky, Anderson and Chambers, 1941). Rigidity of the cell wall was also demonstrated in *Spirillum volutans* (King and Beams, 1942). The cells of this organism do not break up, nor are they greatly distorted by ultra-centrifuging. The intracellular inclusions are concentrated in the "centrifugal troughs" of the body, and the centripetal "crests" appear homogeneous. Elasticity of the cell wall was also shown by microdissection (Wámoscher, 1930). Robinow (1945) extended a method previously used by Gutstein (1924) for the demonstration of the ectoplasm: the cells are first stained with Giemsa's solution, mounted in a drop of water and a suitable field photographed; only the cytoplasm is said to be stained. The preparation is then removed, decolorized with acid alcohol, mordanted with 5% to 10% tannic acid, and restained with 0.02% of crystal violet in water; this is said to stain only the cell wall. The same field previously photographed after staining with Giemsa's solution is found, photographed, and the cells compared. The pictures published by Robinow leave more than a passing suspicion that the structures stained by crystal violet represent more than the cell wall. Recently Dyar (1947*a*) developed, in the reviewer's laboratory, a method in which the cells are treated with a cationic surface-active mordant, cetyl pyridinium chloride, the cell wall stained with Congo red, the cytoplasm with methylene blue. According to Dyar (1947*c*), this method was more successful with spirochetes than the two methods described above.

Dyar (1947*c*) determined cell wall resistance to hydrolysis with *n* HCl at 100° C. The wall of *Bacillus cereus* required 40 minutes for complete disintegration; those of *Bacillus mycoides* and *Bacillus subtilis* required 30 minutes; that of *Staphylococcus aureus* required 12 minutes and that of *Escherichia coli* 10 minutes.

The Slime Layer. The slime layer may be demonstrated with Knaysi's cell wall stain. It appears as a pale red layer of variable thickness surrounding the blue cell wall (Knaysi, 1943*a*). With the electron microscope the slime layer is not usually visible, but may be demonstrated when the cell suspension is prepared in a small droplet of water directly on the collodion film, or when a suitable mordant is used. Under these conditions the slime layer is better preserved and its density is increased (Mudd, Heinmetz and Anderson, 1943*a;* Knaysi, 1944). In homologous serum the slime layer binds some components of the serum and increases both in density and in thickness (Mudd, Heinmets, and Anderson, 1943*b*). The swelling reaction of the pneumococcus was quantitatively studied (Mudd *et al.,* 1943*b:* Johnson and Dennison, 1944). Under special nutritional conditions the thickness and density of the slime layer may be so increased that its demonstration with the electron microscope becomes immediate, and sometimes it may entirely mask the cell.

With respect to chemical composition the slime layer may be of two general types. The first is nitrogen free, as in *Rhizobium leguminosarium* and *Klebsiella pneumoniae;* the second is nitrogen-containing, as in *Bacillus anthracis* (Kramár, 1921). Capsular material is made up of large complex molecules; in the pneumococcus these molecules are filamentous. Within either type there is variation, not merely in spatial configuration, but also in the products of hydrolysis. Indeed either type may be found in different strains of what is considered the same species (Heidelberger, Kendall, and Scherp, 1936). The capsule of *Bacillus mesentericus* and of *Bacillus anthracis* (Ivanovics, 1938, 1939; Hanby and Rydon, 1946) and the extracellular slime of *Bacillus subtilis* (Bovarnick, 1942) are said to be polypeptides of d-glutamic acid.

It was suggested (Etinger-Tulczynska, 1933; Mudd, Heinmetz, and Anderson, 1943*a*) that a distinction should be made between true capsules and extracellular slime. The slime layer is not an integral part of the cell; its formation is considerably affected by the environment (Ivanovics, 1937; Morgan and Beckwith, 1939; Hoogerheide 1939, 1940; also a number of personal observations), and it may be removed without fatal injury to the cell (Dubos and Avery, 1931; Sickles and Shaw, 1933; Shaw, 1937; Dubos, 1939; McClean, 1941, 1942). Bacterial strains without a demonstrable

slime layer may be induced to form large capsules by dissociation procedures (Knaysi, 1933).

Medical Aspects. In pathogenic organisms the cell wall and slime layer protect the bacterial cell against body fluid and are thus factors in virulence; the binding by the capsule of *Micrococcus pneumoniae*, and by the cell wall of *Eberthella typhosa*, of components of the homologous serum (Pijper, 1938, 1941; Mudd and Anderson, 1941; Mudd, Heinmets and Anderson, 1943*b*) is direct evidence for that action. It was also shown that removal of the capsule gives some protection to the animal. The cell wall and slime layer are important in diagnostic procedures, since the serological specificity of bacterial strains and the antigens responsible for agglutination are said to be located at the surface of the cell. In the words of Mudd (1944), "Type specific serologic reactivity, on which specific antibacterial immunity depends, may be determined by configuration either of a carbohydrate or of a protein component. The capsular polysaccharides of the various pneumococcus types are the classic examples of carbohydrate antigens which determine type. The surface somatic proteins ('M substance') of the various types of *Streptococcus* pyogenes are an example of type determination by protein antigens. In the case of the typhoid-paratyphoid-dysentery group, type specificity is determined by a phosphorus-containing polysaccharide-protein complex in which the carbohydrate component is not type specific. The characteristic antigen reactivity (and toxicity) of the somatic antigens of the group appear, however, to be a property of the intact phosphorus-containing polysaccharide-protein complex and not of either the isolated carbohydrate or protein component alone. In all cases known to me, however, the reactive configurations which determine type specificity and antibacterial immunity are peripheral, *i.e.*, are in the capsule, as in pneumococci, in the bacterial cell wall, as in *Streptococcus pyogenes* and the Shigellas, or in the bacterial cell wall and flagella, as in *Eberthella typhosa* and the Salmonellas." A review of the literature on this phase of cyto-immunology may be found in the paper by Mudd from which this quotation was taken and in Dubos (1945).

Physico-Chemical Analysis of the Cell Surface. Electrophoretic mobility has long been considered a function of the cell surface, and

Abramson, Moyer and Gorin (1942) recommended use of the microscopic method for studying the surfaces of living cells. Dyar and Knaysi (1947) showed that the electrokinetic potential of the bacterial cell is an exclusive property of its surface and is not affected by change in the composition of the protoplasm.

Moyer (1936) found that electrophoretic mobility in young cells of *Escherichia coli* was lower than in mature cells; the mobility of the smooth strains remained constant throughout a long range of pH, whereas that of the rough strains varied with the pH. He also noted that the changes of mobility of rough or smooth strains with the concentration of electrolytes were similar to those of some carbohydrate and lipoid surfaces. Stearns and Roepke (1941) observed differences in mobility between rough and smooth strains of *Brucella abortus* similar to those observed by Moyer in *E. coli.* Bradbury and Jordan (1942) concluded from electrophoretic measurements that sulfanilamide and related drugs behaved, at the cell surface of *E. coli,* as did *p*-aminobenzoic acid, and that the drugs associated with the cell through their aromatic amino groups. Cohen (1945) detected imidazole and amino groups at the cell surface of *Proteus.* Dyar and Ordal (1946) compared the electrokinetic behavior of representatives of various groups of bacteria, a myxococcus and a spirochete, with the behavior of known particles. They concluded that the chemical nature of a substance present at the cell surface may be detected by its electrokinetic behavior in the presence of surface-active agents. In the reviewer's laboratory Dyar (1947*b*, 1947*c*) found that the "lipids present in the surfaces of living bacterial cells can be measured somewhat quantitatively. The amount of lipid in the surface of cells of *Micrococcus aureus* and *Mycobacterium smegmatis* can be altered at will, within limits, by varying the conditions of growth. Some other bacteria such as bacilli do not produce surface lipids even though the cytoplasm is full of fat. . . . Amphoteric material in the surface of *Micrococcus aureus,* distinct from the lipid, was detected by change of mobility with pH. . . . Some of the properties of the cell wall 'skeleton' of *Micrococcus aureus* remaining after lipase and trypsin treatment were determined. They indicate, but do not prove, that it may be of carbohydrate nature although unlike either cellulose or the insoluble dextran synthesized by some streptococci".

MOTILITY

Motile bacteria may be divided into two groups: those which creep slowly on solid surfaces or the surface film of liquids, and those which swim freely throughout a liquid. Creeping motion is observed only among the large sulfur bacteria and myxobacteria. It is accomplished by periodic deformation of the cell (Ullrich, 1926). Certain peculiarities in the motion of myxobacteria on the surface of agar were observed by Stanier (1942). In the swimming group, cells of the bacteria have filamentous appendages, called "flagella", generally considered the organs of locomotion. The spirochetes are generally considered to move by deformation of the cell. These general conceptions, apparently long established in bacteriology, were recently shaken by the discovery of flagella-like appendages in the spirochete *Treponema* (Wile, Picard and Kearney, 1942; Morton and Anderson, 1942; Wile and Kearney, 1943; Mudd, Polevitzky and Anderson, 1943) and by recent observation of Pijper (1946, 1947*a*) of motile cells of *Eberthella typhosa* apparently without flagella. On the other hand, Lofgren and Soule (1945) reported that the flagella-like extensions they observed in *Spirochaeta novyi* were the result of the mechanical action of distilled water and centrifugation; in the case of bacteria the challenge of Pijper's conclusion that flagella are threads of slime which are the result, not the cause, of motion has not yet been met; on the contrary, the recent observations of Hillier and Knaysi (1947*a*) on the origin of flagella in *Bacillus mycoides* appear to be in partial agreement with the corresponding phase of Pijper's hypothesis. It is not generally known, however, that students of the flagellates do not agree on the specific rôle of the flagella in the motion of these organisms (Metzner, 1920; Lowndes, 1941; Brown, 1945). Regardless of the outcome, Pijper's hypothesis has stimulated thought and research which will doubtless lead to a better understanding of the nature and function of the flagella and of applications in taxonomy and immunology.

Flagella are not necessary for the life of the cell (Lominsky and Lendrum, 1942). They may be lost by dissociation (Neri, 1940), and their formation is somewhat affected by the environment (personal observation). They adsorb antibodies from a homologous serum with a consequent increase in thickness and stiffness, and the formation of loosely held groups upon agglutination (Pijper,

1938, 1941; Mudd and Anderson, 1941). They agglutinate with acid at about pH 4.4 (Malek, 1938; Ogonuki and Miyata, 1940). Their chemical nature was studied by Boivin and Mesrobeanu (1938).

Other papers of interest are that of Ôgiuti (1936) in which the effect of pH, temperature, osmotic pressure, viscosity and homologous serum on the velocity of various bacteria was studied; that of Hutchinson and McCracken (1943) on a sulfur bacterium; that of Johnson, Zworykin and Warren (1943) on luminous bacteria; and that of Hofer (1944) on *Azotobacter.*

INCLUSIONS

Studies with the ultracentrifuge led King and Beams (1942) to conclude that all demonstrable inclusions of *Spirillum volutans* have higher specific gravity than its cytoplasm.

Lipoid Inclusions. It was shown by Knaysi (1945*a*) that the lipoid inclusions formed by *Bacillus cereus* in the presporal stage give the Sharp test for protein a positive Feulgen reaction. More recent observations also showed that they stain like the nuclei, although somewhat more slowly, with Giemsa's solution. This should explain the high specific gravity of the fatty inclusions of *Spirillum volutans* observed by King and Beams. Knaysi observed the formation of these inclusions in the cytoplasmic membrane and their elimination into the cytoplasm. They are not used up when the cell is transferred to a fresh medium, but are distributed in the progenies of that cell. They are formed even in very poor media, and their size is not greatly influenced by the composition of the medium. Burdon (1946) found that "stainable fatty material in the form of cytoplasmic inclusions, or such material associated with structural elements of the cells, is present in all fungi and in the great majority of bacteria, whether these are aerobic or anaerobic, saprophytic or parasitic, pathogenic or nonpathogenic. . . . With certain notable exceptions (*e.g., Alcaligenes fecalis* and *Azotobacter*), fatty material was found to be more abundant and to occur with greater regularity in Gram-positive bacteria than in Gram-negative bacteria. An apparent tendency for saprophytic species to contain more fat than parasitic species was noted. . . . Although media of special composition are required for the appearance of stainable lipid in the case of certain bacteria, the formation of fatty inclusions was shown

not to be dependent upon the presence of glucose, glycerol, or other fermentable carbohydrate in the medium". Burdon observed a remarkable constancy in the location of these inclusions within the cell in a given genus and sometimes in a given species. Knaysi (1946*d*) found that the lipoid inclusions of the yeast *Hansenula anomala* "are noticeably utilizable only by volutin-free cells, in an environment devoid of nutrients, and under strongly aerobic conditions, both as a source of energy and a source of nitrogen". There was no evidence that they were used up during active growth in complete nutritive media; "on the contrary, the rate of their formation in such media is highest during active growth". Starkey (1946) found a definite relation between lipid content of certain soil yeasts and the composition of the medium.

Volutin. Delaporte (1939) showed that volutin is essentially ribonucleic acid. It can be removed from the cell with 0.02% sodium bicarbonate solution, but some protein was always present in the extract. Knaysi (1946*d*) found that the volutin inclusions of *Hansenula anomala* were readily utilized as a source of nitrogen but not of energy. In a glucose solution the volutin is used up and the stainability of the cell is reduced so that the nucleus may be demonstrated by simple methylene blue staining. In complete media volutin accumulates during active growth and is used up in old starving cultures. Knaysi and Baker (1947) found that ribonucleic acid is present in high proportion in the endospore of *Bacillus mycoides*. In a medium containing a source of energy but not of nitrogen, the spores germinate, using up their supply of ribonucleic acid. The vegetative cells developed have a poorly stainable cytoplasm and are transparent to electrons at ordinary voltages, except for the nuclei which become readily demonstrable by simple staining or with the electron microscope. Knaysi (1947*a*) showed that cells thus developed are highly permeable. Apparently the loss of ribonucleic acid from the cytoplasmic membrane loosens the structure of that membrane. Cells grown in ordinary media contain ribonucleic acid diffuse in the cytoplasm, and probably present in the cytoplasmic membrane, which is responsible for the uniform staining of these cells and their uniform opacity to electrons. The cells of *Bacillus mycoides* do not usually deposit volutin, and the ribonucleic acid is probably present in combination with other protoplasmic constituents, since it is not re-

movable with mild alkalis. It is removable by hydrolysis with *n* HCl, as in the Piekarski-Robinow technique (Vendrely and Lipardy, 1946). Knaysi (1942) had shown that volutin does not interfere with the Feulgen reaction because it is removed by hydrolysis with *n* HCl.

VEGETATIVE REPRODUCTION

In young cultures and in ordinary media, normal common bacteria reproduce almost always by a process known as "cell division". In old cultures, and in young cultures under special conditions, budding may be rarely observed. Frequent budding was observed by the reviewer in *Azotobacter indicum* and in a pneumococcus strain. Cells which have undergone considerable change of form and size may reproduce by fragmentation, a process which is a mixture of aberrant cell division and budding.

Cell Division. Details of the process of cell division were observed by Knaysi (1941*a*). A ring grows from the cytoplasmic membrane inwards, separating the cytoplasm into two parts. This cell plate is similar to the cytoplasmic membrane in chemical and physical properties. The cell plate is then split, apparently inwards. Sometimes the separation is not complete, and the sister cell plates may still be attached in the center; in dried cells, shrinkage stretches this attachment into a plasmodesma. Separate cell walls are formed, leisurely, for both sister cells. In typical cases division is equational. However, asymmetric cell division is occasionally observed in young culture and more frequently in old ones. Knaysi (1933) developed a strain of *Bacillus megatherium* in which asymmetric cell division was strikingly common in young cultures.

Description of the process by Robinow (1945) is very similar to that given by Knaysi (1930, 1941*a*, 1944). The only difference is that Knaysi stated that a cell wall is formed independently by each sister cell, and Robinow (1945, p. 376) was not sure whether the cell wall is "double from the beginning or first laid down in single thickness". Therefore, the statement: "Knaysi . . . concluded . . . that bacteria divide by a retraction of the cytoplasm in opposite direction from the middle plane" (p. 371) must be overlooked. Division by "retraction of the cytoplasm" had been described earlier by Knaysi (1929) for *Mycobacterium tuberculosis,* but his later studies (1941*a*) showed that this organism divides like other bac-

teria. In *Schizosaccharomyces pombe* also Knaysi (1941*a*) had found that the cytoplasm is directly split by the formation of cell walls without previous formation of a cell plate by inward growth of the cytoplasmic membrane, as in the bacteria, and Robinow really confirmed this observation by stating: "Cytoplasmic cell boundaries or cell plates which, in bacteria, are usually laid down before the formation of transverse cell walls were not observed in the fission yeast". It is indeed incomprehensible why, a few lines before, Robinow wrote: "For a different opinion see Knaysi (1941)". This difference between the division of bacteria and yeasts was one of the chief points reported for the first time, made clear and repeatedly emphasized in Knaysi's paper. Recent unpublished observations on the smooth strain C_2 of *Bacillus mycoides*, made by Knaysi and Hillier with the electron microscope, show that the centripetal split of the cell plate is usually brought about by centripetal growth from the lateral wall, and that the new cross wall is differentiated into two layers often before its growth is complete. Severance of the sister cells is apparently brought about by the pressure resulting from their growth and superficially simulates simple constriction.

Budding. No detail study of budding in bacteria was made. The reviewer's preliminary observations on *Azotobacter indicum* indicate that it proceeds from a broad base, often suggesting a process intermediate between budding and cell division.

Fragmentation. By fragmentation is meant the apparently irregular break-up of a cell into smaller ones with or without simultaneous or subsequent growth. The break-up may be accomplished by a cytological process indistinguishable from cell division, as in *Bacillus megatherium*, or by a combination of division and budding, as in the large bodies of Dienes.

The "Large Bodies" of Dienes. In a series of papers Dienes (1939, 1940, 1942, 1943, 1944, 1945, 1946, 1947) described what he considers a complex reproductive process in bacteria, particularly in Gram-negative ones. It consists of "the swelling of bacteria into large, round bodies and the reproduction of bacteria either directly in them or by the intermediary of a soft granular growth (L_1) . . .". This process was observed chiefly in freshly isolated pathogenic bacteria in cultures or when cells from the proper strain were transferred to water. In *Proteus* the process was observed

regularly "when spreading cultures of appropriate strains come in contact". No union of cells was involved. Dienes suggests that this may indicate a sexual process. Dienes and Smith (1944) observed that in a strain of *Bacteroides* "The bacterial filaments segment and the large bodies develop from the neighboring ends of two segments. This process suggests an autogenous conjugation, examples of which are well known in fungi".

It is not possible at the present time to give this process a final interpretation. The formation of normal bacterial forms or L_1 forms from the large bodies does not seem to take place by what is known as "free cell formation", but by budding and fragmentation. This much is clear from the series of observations published by Dienes and particularly from the slides shown by Smith, Hillier and Mudd (1946). The reviewer is convinced that the swelling of bacterial cells is always associated with a change in permeability and with osmotic relationships. When *Bacillus mycoides* is grown in glucose broth containing 0.5 molar $MgSO_4$, numerous swollen cells, similar to the "large bodies" of Dienes, are observed, particularly during an early period of cultural development. At later stages these swollen cells, known as "plasmoptysis figures", are reduced in number, and normal cells predominate. According to Stapp and Zycha (1931), the swollen cells are unable to reproduce. The reviewer's observations with *Escherichia coli* are of special interest. When the cells are transferred from an ordinary medium to one containing 0.25 to 0.50 molar $MgSO_4$, they are at first plasmoylzed; many of the cells are subsequently deplasmolyzed and grow, forming a highly pleomorphic culture consisting of thick curved cells with abruptly tapering ends, of ring forms and of swollen cells of various forms, resembling a culture of a pleomorphic yeast. All types of cells are vacuolated. The swollen cells are often irregularly partitioned, and budding is frequently observed; the explosive separation of a segment and its reunion with the mother cell were sometimes observed, indicating softness or possible absence of a cell wall. In certain cases one gains the impression of the break-up of one of the giant cells into smaller irregular ones, but this was not actually observed. Dienes also observed the formation of "large bodies" in response to toxic influences, but these did not reproduce. The reviewer's observations indicate that enlargement of the cells is not due to mere swelling, but chiefly to actual growth.

In view of the close similarity between these observations and those of Dienes', the phenomenon may be considered a response to the environment. The influence of one *Proteus* strain on another may be readily attributed to mutual antagonistic influences. Sexual stimulation without actual fusion is known in the lower fungi, but such an interpretation should be postponed until more is known about the cytological processes involved. The distinction between large bodies "normally" formed and large bodies formed in response to toxic influences on the basis of ability to multiply may not be desirable, since the giant cells of *Escherichia coli* formed in the presence of $MgSO_4$ show a tendency to multiply by forming irregular plates. The difference may be in the magnitude and nature of the "toxic" influence. The morphologic peculiarities of the L cells is due to the manner in which they are formed and to softness of the cell wall; it seems that when the cell is deformed as the giant cells are, polarity of the cell is affected or temporarily lost, and it reproduces vegetatively by forming plates of division in indefinite directions and breaking up into smaller pleomorphic units. These units should not be called "gonidia", since the term, as now understood, involves free cell formation.

THE ENDOSPORE

Cytology of Formation. *a) Growth from a single granule.* This was described by Hoyt (1935) in *Clostridium septicum,* but examination of his photographic record indicates that his granules may well have been forespores formed by "condensation of the protoplasm".

b) Condensation of the protoplasm. This includes those methods in which the endospore is said to be initiated by the sudden appearance, in a previously differentiated part of the protoplasm, of a primordium or a forespore with dimensions exceeding, equalling or slightly below those of the mature spore. It was described by Delaporte (1934, 1935, 1936) in *Oscillospira guilliermondi* and *Oscillospira batrachorum* of the Cyanophyceae, and in *Spirillum praeclarum;* by Roberts (1942) in *Clostridium pasteurianum;* and by Knaysi (1946*c*) in *Bacillus cereus.*

Nuclear Behavior. Allen, Appleby and Wolf (1939) observed three methods of spore formation in a bacillus isolated from grass. "The main changes involved are explained on the assumption that

the normal vegetative cell is haploid and contains one chromosome. Prior to one type of spore formation a diploid cell is thought to be formed by division of the chromosome, accompanied by a failure in cell division. Meiosis there occurs, resulting in the formation of a well-marked, deeply staining tetrad, followed by separation of the chromosomes and possibly a second mitosis. One of the resultant chromosomes is involved in the formation of the spore, and the remainder appear to be extruded. Klieneberger-Nobel (1945), using the Piekarski-Robinow technique, confirmed the observations of Badian (1933, 1935) which, like those of Allen *et al.*, attribute to the vegetative cell a haploid state followed by autogamy and two nuclear divisions (reduction division). Only one of the resulting chromosomes forms part of the spore; the other three disintegrate.

Knaysi and Baker (1947) observed with the electron microscope spore formation in cells of *Bacillus mycoides* made transparent by growth in a nitrogen-free medium. Their observations suggest that the nuclei of the potential sporangium gather into two groups of one to three nuclei each. The two groups are formed at a certain distance from each other, and an area, slightly denser than the cytoplasm of the mother cell, appears to grow from each nucleus, or groups of nuclei, toward the other; the forespore results from the merging of those two areas, and its length is determined by the relative position of the two nuclear groups. In non-filamentous growth no nuclei were left out to disintegrate in the sporangium, and there was no evidence of nuclear fusion before or after the formation of the forespore, although the authors do not exclude the possibility. At first the nuclei occupy polar positions, but later may be seen anywhere in the spore.

In the light of this research it seems that Badian and his followers were confused by the presence of presporal inclusions. In *Bacillus cereus* these inclusions originate in the cytoplasmic membrane and are eliminated to the cytoplasm. They give a positive Feulgen reaction and take nuclear stains, although more slowly than the nuclei (Knaysi, 1945*a*, 1947*b*). Imšenecki (1945) observed lipoprotein granules in all aerobic sporeformers and stated that under certain conditions these granules stain with basic dyes and have been mistakenly considered nuclei.

Structure of the Endospore. Delaporte (1939–40) observed a refractile membrane, a hyalin cytoplasm and a Feulgen-positive rod or granule. Robinow (1945) concluded from observation of hydrolyzed spores of various bacilli that the endospore consists of "three concentric layers. . . . The central portion, a narrow core, is refractile and stainable, and merges into a thick layer of less refractile and less readily stainable cytoplasm, bounded on its outer surface by a thin crust of stainable, non-refracting material which may be identical with the spore membrane. . . . No chromatinic matter is found in the cytoplasm". He described a biconcave nucleus, "the most refracting element in hydrolyzed spores; it is attached to the outer surface of the cytoplasm." Stille (1937) had observed a single Feulgen-positive body in the endospore and stated that the youngest vegetative cell contains two or four similar bodies.

The work of Knaysi (1945*d*, 1947*b*) with the light microscope and that of Knaysi and Baker (1947), Knaysi, Baker and Hillier (1947) and Hillier and Knaysi (1947*b*) with the electron microscope show that the endospore of *Bacillus mycoides* consists of a double membrane, an inner rigid one and an outer elastic one, enclosing a cytoplasm rich in ribonucleic acid and in lipoidal material diffuse throughout; the nuclei, usually two or more, most commonly occupy polar positions. The reviewer has been able to satisfy himself that the shape of the nucleus and its extra-cytoplasmic position observed by Robinow are artifacts caused by a combination of incipient germination and hydrolysis. Recent work (Knaysi and Hillier, 1947) with *Bacillus megatherium* showed that this organism sheds a single coat consisting of long, beaded, parallel molecules cemented together by a substance which disappears soon after germination. It is not known whether or not an inner coat existed in the resting spore and was used up during germination.

Cytology of Germination. Lamanna (1940) made a careful study of spore germination in *Bacillus* and found correlation between the mode of germination, spore antigen and several physiological properties. Knaysi and Gunsalus (1944) confirmed Lamanna's work with the Marburg strain and the Ford and Lawrence strain of *Bacillus subtilis;* the latter strain was renamed *Bacillus licheniformis* by Gibson and Abdel-Malek (1945). Details of spore germination in *Bacillus mycoides* were studied with the electron microscope by Knaysi, Baker and Hillier (1947); the spore is

surrounded by two coats which become bent and split equatorially. Lateral splitting of the spore coat was recently observed by Knaysi and Hillier (1947) in a strain of *Bacillus megatherium* heretofore considered to germinate by absorbing its coat. Only one coat is shed. The possible existence of a second coat and its utilization during germination are considered, but no evidence for the existence of such a coat has yet been found.

Antigenic Structure. Krauskopf and McCoy (1937) found a close serological relationship between spores and vegetative cells. On the other hand, Lamanna (1940*b*, 1940*c*, 1942*b*), Howie and Cruickshank (1940) and Bekker (1944–45) were able to demonstrate antigens characteristic of the spores, and Lamanna was able to classify members of the genus *Bacillus* on the basis of their characteristic spore antigens. The observation of Dyar in the reviewer's laboratory that spores have a different electrophoretic behavior from vegetative cells is a confirmation of the existence of surface antigens characteristic of the spores.

Chemical Composition. Little is known about the chemical composition of the endospore other than that it contains a number of enzymes (Effront, 1914; Ruehle, 1923; Cook, 1931; Tarr, 1933; Virtanen and Pulkki, 1933) and that it contains more bound water and less free water than the vegetative cell (Friedman and Henry, 1930; Henry and Friedman, 1937). Curran, Brunstetter and Myers (1943) demonstrated Ca, K, Cu and Mn by spectrometric analysis. Knaysi (1945*d*) demonstrated ribonucleic acid diffuse throughout the cytoplasm and utilizable as a source of nitrogen and phosphorus but not for energy. It may be added that the cytoplasm of the spore is rich in lipoid material, also diffuse. Since the spore contains nuclei, it also contains the nucleoproteins characteristic of the nucleus.

Effects of the Environment on Spore Formation. Although the endospore is a part of the life cycle of certain bacteria, its formation is considerably affected by the environment. Loss of the ability to form endospores, with or without regaining that property, was reported by Knaysi (1933, 1935*a*); the effect of dissociation on the relative number of spores in a given culture was studied by Hayward, Marchetta and Hutton (1946). The environmental factors investigated during the last decade were the following: the temperature of incubation, composition of the medium with respect to

type and initial concentration of gross nutrients, molecular oxygen, salts, pH, nutrilites; the accumulation of by-products; gradual drying of the medium; position of the cell in the colony (Greene, 1938; Kaplan and Williams, 1941; Roberts and Baldwin, 1942; Hayward, 1943; Knaysi, 1945*c*; Knaysi and Baker, 1947). It was shown by Knaysi that these factors are not independent variables but are interrelated by their specific effect on the utilization of food and the health of the cell. Knaysi concluded that, with *Bacillus mycoides,* spores are formed by healthy cells facing starvation, and the ideal environment for sporulation is that in which food is completely utilized without accumulation of by-products. Whether or not there are organisms stimulated to sporulate by the accumulation of metabolic products remains to be shown. Sporulation has an optimum temperature and a sharp optimum pH; it does not usually require nutrilites other than those required for growth, but nutrilites may exert an indirect stimulating effect; for instance, vitamin B_1 may promote sporulation, in a medium where acid products of metabolism tend to accumulate, by its decarboxylic activity which prevents a significant drop in pH. However, it was recently reported by Dutky (1947) that he developed several media for the growth of *Bacillus popilliae* and *Bacillus lentimorbus;* these media "gave consistently high yields of vegetative forms, but none was adequate for sporulation". Whether this indicates need for an additional growth factor is not clear. Drying of the medium is detrimental to sporulation by *Bacillus mycoides* except when drying significantly increases oxygen supply.

An interesting effect of the temperature of incubation was reported by Christian (1931) on a form of *Bacillus* isolated from heated milk. The optimum temperature for the growth of this organism was between 30° and 37° C. and the maximum for its sporulation 22° C. At 37° C. the organism shows "evidence of an effort at spore formation. The cell contents concentrate in a terminal position, as in the case of the formation of true spores, but no spore wall is formed. Later, these terminal bodies or 'abortive spores' are liberated and persist in the cultures in the free state for long periods. It has not been demonstrated whether they are capable of developing further under suitable conditions or of giving rise to new generations of the organism". One wonders whether or not similar bodies recently observed in certain thermo-

philic bacteria (Buck, 1947, and personal communication) are abortive spores which may attain full development below the optimum temperature for vegetative growth.

Effect of the Environment on Spore Germination. Structurally and chemically, the normal endospore is a complete cell, and when it is placed in a suitable environment, it proceeds to germinate. However, the time it takes a given spore to germinate varies considerably with the environment; in a given environment spores of a given culture do not all germinate at the same time. Delayed germination or dormancy observed chiefly among heated spores, and to a certain extent in unheated spores, is probably due to injury or deficiency, and the number of "dormant" spores decreases as the quality of the medium in which they are planted is improved (Morrison and Rettger, 1930; Curan and Evans, 1937; also personal observations). Indeed a normal spore may germinate in an environment containing the necessary nutrient even when this environment is otherwise detrimental to the survival of the germ cell (Knaysi, 1945*d*).

With respect to concentration of food Curran (1931) found that in peptone solutions the minimum concentration for germination is 0.025%; in 10% peptone there is considerable retardation of germination. Knaysi (1945*d*) observed no germination of *Bacillus mycoides* in distilled water or in solutions of various salts, but there were germination, growth and spore formation in bacto agar prepared with distilled water without the addition of any nutrients. Germination was also observed in a solution of 0.2% glucose and 0.1% potassium phosphate, but not when the glucose was replaced by lactose, a sugar not fermented by the organism. It was concluded that the endospore contained a relatively large quantity of a substance which is an excellent source of nitrogen but no source of energy. Knaysi and Baker (1947) identified the substance as ribonucleic acid and showed that germination can take place in a solution of glucose buffered with Na acetate, indicating that ribonucleic acid is also a good source of phosphorus. It results from this work that there is no minimum concentration of the nitrogen or phosphorus sources which should be added to the medium, and that the minimum for the source of energy is below 0.2% of glucose. Evans and Curran (1943) and Curran and Evans (1945) showed that germination is favored by exposure of the spores to "sublethal

heat". Knaysi (1947*a*) observed that germination in a glucose solution is favored by previous autoclaving of the sugar solution or by the addition of traces of phosphate. This effect seems to consist of activation of the sugar and is expressed by rendering reversible and by lowering of the oxidation-reduction potential (Wurmser, 1930; Knaysi, 1935*b*); autoclaving of culture media, or the addition of reducing substances, is known to be beneficial for the initiation of growth (Dubos, 1929; Allyn and Baldwin, 1930, 1932; Wood, Wood and Baldwin, 1935; Nelson, 1944). The beneficial effect observed when the spores are heated in distilled water may be attributed to activation of material inside of the spore.

Consequently the behavior of a normal spore in a given environment is better understood when one realizes that it is a cell, resting because of the lack of food or of the proper conditions for its utilization, containing a source of nitrogen, minerals and enzymes, but separated from its environment by a coat of low permeability.

Resistance of the Endospore. From the practical point of view the chief characteristic of the endospore is its ability to attain a high degree of resistance to drying, heat, chemicals and other deleterious agents. It should be emphasized, however, that the resistance of endospores varies considerably with the species; nevertheless, it is not a constant for the species. It varies considerably with the conditions under which the spores were formed (Theophilus, 1937) and in the same culture varies considerably from spore to spore (Hodge and Knaysi, 1937); a morphological basis for variation in resistance between spores of the same culture was supplied by Knaysi, Baker and Hillier (1947). The cause of spore resistance has been a subject for speculation. In the early decades of bacteriology it was attributed to the existence of a thick coat of low permeability. Later investigators attributed resistance to peculiar spore enzymes and other proteins or to a low water content. The rôle of the spore coat in resistance to chemicals can not be denied; it can be deduced *a priori* and may be concluded from the observation of Knaysi (1945*d*) that when glucose is fermented by spores, the pH reached is considerably below that reached when vegetative cells are used; that the fermenting spore gradually assumes vegetative characteristics without normal germination and is killed by the hydrogen ions. Some correlation was also found between toughness of the spore coat and resistance to moist heat

(Knaysi, 1938; Lamanna, 1937). The high resistance of the spores of thermophilic bacteria to heat (Esty and Williams, 1920) is undoubtedly due chiefly to peculiar enzyme systems and other proteins stable at high temperature; a correlation was also found by Lamanna (1942*a*) between the maximum temperature of growth and the resistance of spores to heat. There is no evidence, however, that in the same strain the spore enzymes, for instance, are different from those of the vegetative cell. Since the spore contains less free water than the vegetative cell (Henry and Friedman, 1937), this difference is probably an important factor in spore resistance to heat as well as to chemicals. In the reviewer's opinion the high concentration of ribonucleic acid and of lipoid material in the cytoplasm of the spore, the low free-water content of the spore and the low permeability of the coat, taken together, should be sufficient to explain spore resistance to drying, heat, chemicals, ultraviolet radiations and possibly other physical and chemical agents. (See also von Angerer, 1938.)

SEXUAL REPRODUCTION

Existence of a sexual mechanism in bacteria has been claimed by a number of bacteriologists (Schaudinn, 1902, 1903; Mellon, 1925; Löhnis, 1921; Enderlein, 1925; Hadley, 1928; Badian, 1933, 1935; Allen *et al.*, 1939; Klieneberger-Nobel, 1945; and many others). The evidence has never been satisfactory. Sherman and Wing (1937) developed substrains of *Escherichia coli* which differed from the parent strain in the ability to ferment certain substances. A strain which was sucrose +, salicin –, and one which was sucrose –, salicin + were inoculated into the same test tube and incubated together for one week. Strains which resembled the parent strains and others which were sucrose + and salicin +, sucrose – and salicin – were isolated from the mixture. However, since similar strains were also found without mixing of the strains, the authors refrained from giving their results sexual interpretation. Gowen and Lincoln (1942), using *Phytomonas stewartii,* drew a similar conclusion.

Recently, Lederberg and Tatum (1946) and Tatum and Lederberg (1947) performed fundamentally similar experiments with *Escherichia coli* and concluded that "Evidence has been presented for the occurrence of character recombination. . . . This suggests

the existence of a sexual phase. Recombination of genes controlling several growth factor requirements and resistance to a specific bacteriophage have been found". The authors admit an alternative interpretation, assuming "the occurrence in the medium of transforming factors capable of inducing the mutation of genes, bilaterally". However, "Attempts at the induction of transformations in single cultures by the use of sterile filtrates have been unsuccessful. . . . The fusion presumably occurs only rarely, since in the cultures investigated only one cell in a million can be classified as a recombination type. The hypothetical zygote has not been detected cytologically". The evidence does not appear to us to be of a higher order than that presented by Dienes and Smith (1944) and by Dienes (1946).

STAINING

The Gram Stain. The structural, chemical and physical bases of the Gram reaction were discussed by Knaysi (1944). Dubos and Macleod (1938) reported that treatment of the heat-killed, Gram-positive cells of pneumococci with a polynucleotidase obtained from animal tissues rendered those cells Gram-negative. Henry and Stacey (1943, 1946) secured the same results with yeasts and several Gram-positive bacteria by the action of certain carbohydrate-splitting enzymes or by extraction with a solution of bile. Extraction of Gram-positive cells with a 2% aqueous solution of bile removes some carbohydrate and a substance identified as Mg-ribonucleate, leaving a Gram-negative skeleton rich in basic protein material. If this skeleton is treated with formaldehyde or certain other reducing agents and soaked in the extract, the Mg-ribonucleate recombines with the skeleton and the cells become Gram-positive again. Henry and Stacey concluded "that the essential constituent in Gram-positive organisms . . . lies in their having as part of their surface structure the magnesium salt of ribonucleic acid". Knaysi (1943*a*) showed that the volutin present in the vacuole of *Thiobacillus thiooxidans* was Gram-positive; volutin is known to consist of ribonucleic acid. Dubos (1945) was able to reverse the Gram reaction of pneumococci by treatment of the heat-killed cells with a solution of crystalline ribonuclease. Bartholomew and Umbreit (1944) confirmed the findings of Henry and Stacey and of Dubos. Bartholomew (1946) found that *Neisseria catarrhalis* was different from other Gram-negative bacteria by becom-

ing Gram-positive when treated with extracts containing Mg-ribonucleate. Knaysi (1947*a*) observed that the vegetative cells of *Bacillus mycoides* developed from the spores in nitrogen-free media were Gram-negative except the nuclei. Dyar and Knaysi (1947) found that when a slant of meat infusion agar is inoculated with a spore suspension of *Bacillus mycoides,* the germ cells and subsequent generations of vegetative cells during the first few hours are, in the majority, Gram-negative.

In view of the observations reported above, it may be asked whether or not it should be concluded that the mechanism of the Gram reaction is exclusively chemical and bound with Mg-ribonucleate, or whether permeability also plays a rôle. Since removal of ribonucleic acid changes the permeability of the cell, even of living cells developed in a nitrogen-free medium, it can not be concluded that permeability does not play a rôle in the Gram reaction. On the other hand, in cells partially autolyzed the cytoplasmic membrane remains strongly Gram-positive. Also when the cells are broken up mechanically, or when the cytoplasmic membrane is partially removed by chemical action, fragments of this membrane remain strongly Gram-positive, although the cytoplasm becomes Gram-negative. It seems, therefore, that the Gram reaction, as applied to the entire cell, is a property of the cell. It is interesting that Henry and Stacey stated that Mg-ribonucleate confers Gram-positiveness only when it combines with complex molecules of the cell surface, meaning the cytoplasmic membrane. It seems that ribonucleic acid exerts both a direct chemical action and an indirect action by affecting permeability of the cytoplasmic membrane. It may also be added that, although ribonucleic acid is the chief substrate of the Gram reaction, the fact that the nuclei of cells developed in a nitrogen-free medium are also definitely Gram-positive indicates that other cell material may contribute to the reaction in the complete cell.

The Acid-fast Reaction. Reeves and Anderson (1937) and Stodola, Lesuk and Anderson (1938) isolated from the "unsaponifiable wax" of the tubercle bacillus a hydroxy methoxy acid which was strongly acid-fast. This acid was named "mycolic acid". Yegian and Porter (1944) showed that "non-acid-fast forms appearing in stained smears of young cultures of the tubercle organism are created by mechanical action of the platinum loop or the

spatula used to disperse the organisms" and that sectioning may also destroy acid-fastness. Yegian and Baisden (1942) and Porter and Yegian (1945) studied the factors which affect beading in the tubercle bacillus and concluded that the Much granules and beads are artifacts. Lamanna (1946) studied the mechanism of beading in mycobacteria stained with Ziehl-Neelsen's carbol-fuchsin and concluded that it is "the result of phenol and dye separating out as a liquid phase" and that "the basis for the acid-fast property lies in the greater solubility of the phenol and dye in the cell constituents than in the decolorizing agent".

CYTOLOGY AND CHEMOTHERAPY

The importance to medicine of a sound knowledge of the structural make up of the bacterial cell and of the properties and behavior of these structures has been realized by a number of eminent bacteriologists (Mudd, 1944; Dubos, 1945; Pijper, 1947*b*), and a number of medical relationships were briefly indicated in previous sections of this review. Of these relationships none has greater potentialities in practical application than those of structure to virulence and resistance of the bacterial cell. In the field of chemotherapy emphasis is placed on metabolic processes, and little consideration is given to the intimate relation between metabolic activity and structure; if loss of the capsule reduces the virulence of pneumococci, then it may be possible to alter the environment so that capsular material is not synthesized. Since some of the permanent structures of the cell do not have a fixed chemical composition, it should be possible by altering the environment to modify the chemical and physico-chemical properties of these structures so that the virulence and resistance of the cell are decreased. When the endospores of *Bacillus mycoides* germinate in a nitrogen-free medium, the vegetative cells which develop are Gram-negative and very permeable to neutral red, even in very dilute solutions. In these cells the cytoplasm and the cytoplasmic membrane are altered in chemical composition; they are practically free of ribonucleic acid and possibly of other substances. Loss of ribonucleic acid from the cytoplasmic membrane seems to increase its permeability; loss of that substance from the cytoplasm should deprive the nucleus and other important components of the protoplasm from a considerable measure of protection against drying, chemicals, ultraviolet light

and other harmful agents; the cells should be more readily killed by components of animal fluids and by chemotherapeutic agents. In this case synthesis was prevented by deficiency of the medium. If synthesis can be partially or totally inhibited by the addition of some substance to a nondeficient medium, then great strides toward the control of disease would have been made. In particular we wish to focus attention on ribonucleic acid. This substance is widely distributed among microorganisms in the cells, and it often precipitates in the form of inclusions (volutin). Among the bacteria it is usually present in both Gram-positive and Gram-negative species; even bacteria which do not normally deposit volutin may contain ribonucleic acid in a large proportion. There are indications that the ribonucleic acid metabolism is of less importance in the cells of higher plants and animals than in bacteria, so that an agent which *specifically* inhibits the formation of ribonucleic acid would not be of high toxicity, at least to adult animals, and would considerably increase the susceptibility of the bacterial cell to the action of body fluids or of chemotherapeutic agents. Indeed the action of penicillin on bacteria—formation of cells of abnormal morphology, interference with cell division, tendency to render Gram-positive bacteria Gram-negative (for the literature see the section on form and size)—suggests that penicillin may partly act as such an agent. It seems, therefore, that a rational approach to chemotherapy should begin by a study of the metabolism of the principal phosphorus-containing compounds, particularly that of ribonucleic acid.

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